

A STUDY OF THE CHARACTER AND FUNCTION OF THE
LYMPHOID CELL POPULATION IN THE EPITHELIUM
OF THE SMALL INTESTINAL VILLI OF THE MOUSE.

BY

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ABSTRACT OF THESIS

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Title of Thesis A Study of the Character and Function of the Lymphoid Cell

Population in the Epithelium of the Small Intestinal Villi of the Mouse.

The lymphoid cell population in the small intestinal epithelium of the mouse was studied by light, fluorescence and electron microscopy. The origin and kinetics of this population was examined by autoradiography. The population was found to have the following characteristics. It is composed of morphologically different cell types. The majority of the cells are small or medium lymphocytes, but occasionally, large pyroninophilic cells are observed, and even less frequently plasma cells are found. The cells are situated between the absorptive epithelial cells, and the majority lie close to the basement membrane. In keeping with the low incidence of pyroninophilic cells, fluorescence studies indicated that the majority of cells do not contain large amounts of antibody.

The population is composed of at least two subpopulations with different lifespans, one of which is short-lived and the other long-lived. The origin of these cells is not clear, but the presence of mitotic figures in the epithelium indicates that the population is in part self-replicating. Cell transfer studies suggest that several cell sources can contribute a few cells to the epithelium, and therefore this population is probably complex in its origin. The fate of these cells is equally complex, a few degenerate, some migrate into the lamina propria and the others are presumably lost into the lumen of the intestine.

The function of this population is unknown, but a role in digestion, antibody production or as a bursal equivalent would tend to be excluded by the results. The hypothesis is presented that these cells are involved in cell-mediated immune responses either as a cellular equivalent of the local IgA antibody system, or alternatively, as cell mediators involved in the regulation of tissue growth.

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SUMMARY.

The lymphoid cell population in the small intestinal epithelium of the mouse was studied by light, fluorescence and electron microscopy. The origin and kinetics of this population was examined by autoradiography. The population was found to have the following characteristics. It is composed of morphologically different cell types. The majority of the cells are small or medium lymphocytes, but occasionally, large pyroninophilic cells are observed, and even less frequently plasma cells are found. The cells are situated between the absorptive epithelial cells, and the majority lie close to the basement membrane. In keeping with the low incidence of pyroninophilic cells, fluorescence studies indicated that the majority of cells do not contain large amounts of antibody.

The population is composed of at least two subpopulations with different lifespans, one of which is short-lived and the other long-lived. The origin of these cells is not clear, but the presence of mitotic figures in the epithelium indicates that the population is in part self-replicating. Cell transfer studies suggest that several cell sources can contribute a few cells to the epithelium, and therefore this population is probably complex in its origin. The fate of these cells is equally complex, a few degenerate, some migrate into the lamina propria and the others are presumably lost into the lumen of the intestine.

The function of this population is unknown, but a role in digestion, antibody production or as a bursal equivalent would tend to be excluded by the results. The hypothesis is presented that these cells/

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CHAPTER 1.INTRODUCTION AND REVIEW OF THE LITERATURE.

The function of the lymphocyte population in the epithelium covering the villi of the small intestine is still debatable. In fact, statements of this nature appear to be one of the few points of agreement in the gradually accumulating literature on this subject. It is the purpose of this introduction, firstly to survey the earlier experimental observations on these epithelial lymphocytes, and secondly, to describe the various hypotheses which have been made as to their function.

EXPERIMENTAL OBSERVATIONS.Distribution and Incidence.TABLE 1.1.

Incidence of lymphocytes in the small
intestinal epithelium of various species.

Species	Percentage lymphocyte to epithelial cells	Reference
Human	7.5	Shields <u>et al.</u> (1969)
Human	8.0	Fichtelius <u>et al.</u> (1969)
Mouse	5 - 35	Andrew and Andrew (1945)
Mouse	9.0	Darlington and Rogers (1966)
Mouse	5.0	Fichtelius <u>et al.</u> (1969)
Rat	9 - 10	Fichtelius <u>et al.</u> (1969)
Rabbit	12 - 23	Fichtelius <u>et al.</u> (1969)
Chicken	6.6 - 13.5	Back (1970a; 1970b)

5

Evid. you cite doesn't confirm

life span of months, although others

evid. does, eg. long-term

labelling. X-rays in man

Although lymphocytes are found in non-mammalian species (Beguin, 1904; Blake, 1936; Fichtelius, Finstad and Good, 1969), only studies reported for mammalian species are presented in the preceding table (Table 1.1). From this table it can be seen that in most of the mammals studied, the percentage of lymphocytes to epithelial cells is about 5 - 10 per cent. The exception appears to be the rabbit, in which the percentage is considerably higher (Jordan and Speidel, 1923a; Fichtelius, Finstad and Good, 1969).

Light Microscope Observations.

Several light microscopical studies are reported in the literature, but there appears to be little agreement on either the morphology of the lymphocytes or their precise relationship to the epithelial cells.

Since it is very difficult to discern the cytoplasm of lymphocytes situated in the epithelium, most observations have been limited to studies of nuclear morphology. The majority of the nuclei are spheroidal and the nuclear diameter has been reported as being 3.3 - 8.9 μm (Kelsall, 1946). However, the shape of some of the nuclei can vary considerably and various configurations, suggesting amoeboid movement, have been described (Wolfe-Heidegger, 1939; Kelsall, 1946). Estimates on the number of lymphocytes undergoing nuclear degeneration vary widely; figures ranging from 1 per cent (Andrew, 1965) to 60 per cent (Shields, Touchon and Dickson, 1969) having been reported. Darlington and Rogers (1966) stated that the lymphocyte nuclei in the epithelium at the base of the villus were pale, whereas those nearer the tip were darker staining./

staining. They did not comment on the significance of this observation.

Although there are some lymphocytes situated close to the brush-border of the epithelial cells, the majority of the lymphocytes, approximately 95 per cent, are close to the basement membrane (Kelsall, 1946; Andrew, 1965; Darlington and Rogers, 1966; Meader and Landers, 1966). Some authors have concluded that the lymphocytes are situated between the epithelial cells (Wolfe-Heidegger, 1939; Andrew and Collings, 1946), while others considered that they were intracellular (Andrew and Sosa, 1947; Kelsall and Crabb, 1958; Shields, Touchon and Dickson, 1969). Their actual situation is, however, very difficult to resolve with the light microscope, and the conclusions drawn must therefore be suspect.

The morphological characteristics of some of the lymphocytes in the epithelium, especially amoeboid forms, has led to speculation as to the fate of these cells. Some authors have concluded that the lymphocytes migrate into the lumen of the intestine (Macklin and Macklin, 1932; Wolfe-Heidegger, 1939), whereas others maintain that they return to the lamina propria (Zawarykin, 1883; von Davydoff, 1887). It is, however, difficult to arrive at firm conclusions solely on the results of static studies with the light microscope.

Electron Microscope Observations.

Electron microscope studies on lymphocytes in the intestinal epithelium have followed the same general lines as the earlier light microscope studies, namely, morphology, position in the epithelium, and the question of migration.

Probably the first recorded observation was that of Palay and Karlin/

Karlin (1959). These workers, in their studies of the intestinal epithelium, observed 'intrusive cells' and concluded that they were probably small macrophages. Meader and Landers (1967) found that the lymphocytes in the epithelium were morphologically similar to those in the lamina propria, with the exception that they contained more lysosome-like dense bodies. The lymphocytes in the epithelium showed no signs of degeneration. On the other hand, Andrew (1965) reported that the lymphocytes migrating from the lamina propria into the epithelium became transformed, in that they lost much of their endoplasmic reticulum, and showed a great decrease in the number of mitochondria. However, they were not degenerating.

The use of the electron microscope has enabled a more detailed study of the relationship of the lymphocyte to the epithelial cell to be made. Andrew (1965), from his studies, supported the concept of an intracellular position for the lymphocytes, whereas, Palay and Karlin (1959) and Meader and Landers (1967) stated that the lymphocytes were between the epithelial cells. The latter authors also concluded that the lymphocytes did not enter the lumen of the intestine, but that all migratory movements were between the epithelium and the lamina propria. These conclusions were based on studies of the ultrastructural equivalent of the polarized movement characteristic of lymphocytes as seen by the light microscope (Lewis, 1931); for example the classic hand-mirror configuration and the trailing of cytoplasmic contents at the rear of the cell.

Autoradiographic Studies.

Autoradiographic/

Autoradiographic studies on lymphocytes elsewhere in the body have shown that there are at least two distinct populations of lymphocytes with respect to life span, long-lived and short-lived (Everett and Tyler, 1967). Of the lymphocytes in the bone marrow and the thymus, 95 per cent are of the short-lived variety with a turnover time of 3 - 4 days, while at the other extreme, 90 per cent of those in the thoracic duct lymph appear to be long-lived with a life span of several months (Everett, Caffrey and Rieke, 1964). This difference in life span is reflected in the labelling index following a single pulse of tritiated thymidine, so that in the bone marrow 75 per cent of the small lymphocytes are labelled 4 days after injection (Osmond, 1966), whereas, in the thoracic duct, less than 10 per cent of all lymphocytes are labelled (Everett, Reinhardt and Yoffey, 1960).

Autoradiographic studies on the lymphocytes in the small intestine have been reported by Fichtelius (1968) for the rat, and by Darlington and Rogers (1966) for the mouse. Fichtelius found a mean labelling index of 12.3 per cent, with a maximum of 20 per cent at any time interval. He concluded that many of the lymphocytes migrating into the epithelium were newly formed and that 2 - 3 per cent synthesized DNA in situ. On the other hand, Darlington and Rogers recorded a maximum mean labelling index of 3.46 per cent, and concluded that the lymphocytes are part of a cell population with a mean generation time of 15 - 22 days. Fichtelius' results would appear to suggest that the lymphocytes in the epithelium are part of a cell population with a fairly rapid turnover rate, whereas those of Darlington and Rogers suggest a much slower cell cycle. However, species difference may be responsible for these variations in/

in labelling indices.

Other workers have reported autoradiographic studies on lymphocyte populations which may be related to the lymphocytes found in the epithelium. The following may be of significance. Olson (1969) obtained a labelling index of approximately 50 per cent for lymphocytes in the lamina propria of the small intestine of the guinea-pig, indicating a population in rapid transit through the region. He did not report on the labelling index of the lymphocytes in the epithelium, so that no conclusions can be drawn on the relationship of the lymphocytes in the lamina propria to those in the epithelium. Miller (1964), in his studies of local labelling in the popliteal lymph node of the rat, found that labelled small lymphocytes migrated to the submucosa of the ileum, and that occasionally, small lymphocytes were seen traversing the bowel mucosa. It is interesting to note that Miller used Salmonella antigens to stimulate the popliteal node and it is probable that similar or cross-reacting antigens would be present in the lumen of the intestine. Gowans and Knight (1964) found that large numbers of thoracic duct large lymphocytes, labelled in vitro, migrated to the lamina propria of the ileum, where they were seen as cells with strongly pyroninophilic cytoplasm. In a similar study, Goldschneider and McGregor (1968) found labelled lymphocytes in the lamina propria, and also between the epithelial cells. These findings suggest a relationship between thoracic duct large lymphocytes and intestinal lymphoid tissue.

In summary, the experimental observations outlined in the previous paragraphs show many inconsistencies. Approximately 10 per cent of the cells of the intestinal epithelium are lymphocytes. These cells may/

may be either intracellular or intercellular, show varying degrees of morphological changes including degeneration, and transformation, and may be migrating into the lumen of the intestine or into the lamina propria. They are part of a cell population which appears to have a rapid turnover rate in the rat, whereas in the mouse the rate seems to be slower. The origin of the cells is unknown.

FUNCTIONAL CONSIDERATIONS.

In the previous part of this section, various experimental observations on lymphocytes in the intestinal epithelium have been outlined. Since much of this evidence is indecisive and even contradictory, it is not surprising that a variety of hypotheses have been presented to explain the presence of these cells in the epithelium. Because of the relative lack of specific information favouring any one hypothesis, little attempt has been made to be critical in the following outline, so that evidence mainly in favour of each hypothesis is presented at the expense of much of the evidence against them. The latter will be dealt with elsewhere in the thesis. The following hypotheses are the main ones which have been suggested.

Lymphocyte Regulation.

The active lymphopoiesis in the thymus (Kindred, 1942) and the apparent movement of 3.3×10^8 lymphocytes from the thoracic duct into the blood stream every 24 hours in the dog (Bunting and Huston, 1921) led to speculations on the regulating mechanism which maintained the blood level constant/

constant in the face of this apparently massive influx of lymphocytes into the circulation.

On the basis of histological studies of the intestine, several authors concluded that the lymphocytes seen in the epithelium were in the process of migrating into the lumen (Macklin and Macklin, 1932; Wolfe-Heidegger, 1939), and consequently, regulation of the blood lymphocyte level was attributed to loss through the gastrointestinal epithelium as previously suggested by Bunting and Huston (1921).

More specific studies on this problem have been reported by Ambrus and Ambrus (1959). These workers injected radioactively labelled leucocytes into dogs with Thiry-Vella intestinal loops and analysed perfusates of the loops at various intervals after injection. The appearance of labelled mononuclear cells in the perfusate led these workers to conclude that regulation of the leucocyte level may be by mucosal diapedesis into the lumen of the intestinal tract. However, the lymphopenia which follows gastroenterectomy led other workers to conclude that the intestine was a major source of lymphocytes rather than a site for their destruction (Erf, 1940; Yoffey, 1942). The drastic surgery involved in these experiments would, however, warrant caution in drawing reliable conclusions.

To summarize, the loss of lymphocytes through the mucous membrane of the intestinal tract has been postulated to act as a regulatory mechanism responsible for maintaining a constant blood lymphocyte level, in spite of apparent massive influx of cells from the thymus and the thoracic duct. Although the recent studies of Metcalf (1966) suggesting that the majority of thymic lymphocytes are destroyed in situ, and the work/

work by Gowans and his colleagues (Gowans and Knight, 1964) on the recirculation of lymphocytes would appear to alleviate the need for such a regulatory mechanism, it is not inconceivable that a large number of lymphocytes may still be lost into the lumen of the intestine.

Defence Mechanism.

Although a defensive role has been suggested by several authors (Hellman, 1934; Stenquist, 1934; Andrew and Collings, 1946), the fact that lymphocytes are present in a situation which is very likely to receive external insults is probably the only evidence which has been brought forward to support this hypothesis. Bunting and Huston (1921) suggested that the lymphocytes fixed 'toxins' arising in the lumen of the bowel, but more recent studies (e.g. Kaplan, Coons and Deane, 1950) have shown that bacterial products such as pneumococcal polysaccharide do not fix to lymphocytes to any significant degree. The more specific hypothesis that these lymphocytes may have an immunological function (Andrew, 1965) suffers from a similar lack of support. There is some evidence that lymphocytes from immune donors will 'home' to the intestine of transfused recipients which have intestinal helminth infections similar to those of the donor (Dineen, Ronai and Wagland, 1968). On the other hand, there would appear to be very little difference between the distribution and kinetics of the lymphocytes in the intestinal epithelium of conventional rats and those in germ-free rats (Fichtelius, 1968), the latter being expected to receive much less antigenic stimulation.

In conclusion, although the immunological capacity of lymphocytes in other situations has been well substantiated, there is surprisingly little/

little evidence to support the hypothesis that the lymphocytes in the intestinal epithelium have a defensive or immunological function.

Bursal Equivalent.

The bursa of Fabricius is a lympho-epithelial organ associated with the hind gut of the fowl. Early studies on the effect of removal of this organ on the immune response of the fowl (Glick, Chang and Jaap, 1956) have since led to the concept of thymic-dependent and bursal-dependent immune responses (Szenberg and Warner, 1962; Cooper, Peterson, South and Good, 1966). Neonatal thymectomy results in a reduction in the efficiency of cell-mediated immune reactions such as homograft rejection, whereas, bursectomy is followed by a marked decrease in the ability to produce antibodies (Cooper, Peterson, South and Good, 1966). Neonatal thymectomy in the mammal has similar consequences to those seen in the avian (Arnason, Jankovic, Waksman and Wennersten, 1962), but unfortunately, there is no obvious morphological equivalent of the avian bursa which can be removed to demonstrate bursal-dependent responses.

Attempts to demonstrate a mammalian equivalent of the bursa of Fabricius have been centred on gut-associated lymphoid tissue, and the close association of lymphocytes with the intestinal epithelial cells has led to the concept of a diffuse lympho-epithelial organ analagous to the avian bursa (Fichtelius, 1967). The concept of a diffuse lympho-epithelial organ goes back to the work of von Davydoff (1887) who thought that the lymphocytes in the epithelium were derived from the epithelial cells, a view which is held today for the origin of both thymic (Auerbach, 1963) and bursal lymphocytes (Ackerman and Knouff, 1964).

In/

In addition to the obvious lymphocyte-epithelial cell relationship, other evidence in support of the hypothesis of a bursal equivalent has come from autoradiographic and phylogenetic studies.

Fichtelius (1968) demonstrated a high rate of DNA synthesis by the lymphocytes in the intestinal epithelium and suggested that this might be equivalent to the high rate of DNA synthesis characteristic of bursal lymphocytes (Warner, 1965). Fichtelius and his colleagues have also presented phylogenetic evidence to show the association of lymphocytes in the epithelium with the evolution of antibody production (Fichtelius, Finstad and Good, 1969). The most primitive vertebrates (e.g. Lamprey - Petromyzon marinus) expressing adaptive immunity to relatively few antigens have low numbers of lymphocytes in the intestinal epithelium. Certain fishes (e.g. Paddlefish - Polyodon spathula) which have a well developed plasma cell and antibody producing system have large numbers of lymphocytes. In amphibians, many of the cells are pyroninophilic and some have the morphologic characteristics of plasma cells. This latter observation shows a distinct association of these cells with antibody production.

In contrast to these findings, neonatal thymectomy decreases the number of lymphocytes in the intestinal epithelium suggesting that they may be in fact part of the thymic-dependent system (Fichtelius, Yunis and Good, 1968). Also, the intestinal lymphocyte population in mice does not begin to expand until the second week of life, whereas in the chicken the primary lymphoid organs such as the thymus and bursa are well established at hatching, even before the animal is fully immunologically competent (Fichtelius, Yunis and Good, 1968).

In/

In conclusion, the search for a mammalian equivalent of the avian bursa of Fabricius as a primary lymphoid tissue associated with antibody production has been centred mainly on intestinal lymphoid tissue. There is some autoradiographic and phylogenetic evidence which suggests that the lymphocytes in the epithelium covering the small intestine may be associated with antibody production and hence may be equivalent to the avian bursa as a primary lymphoid organ.

Trophocytic Function.

A trophocytic function for lymphocytes was probably one of the earliest hypotheses put forward in an attempt to ascribe a role for these cells. Ranvier (1875) on the basis of the mobility of the lymphocyte and the presence of glycogen and other granules in the cytoplasm concluded that lymphocytes were 'les glandes unicellaires mobiles'. Since that time, lymphocytes have been postulated to supply nucleoproteins (Medawar, 1957), energy (Bristol, 1919) and the basic elements necessary for protein synthesis (Ehrich and Seifter, 1953) to various cells and organs in the body. However, evidence for a trophocytic function of lymphocytes is practically all indirect and circumstantial (Loutit, 1962). The three main lines of evidence which have been presented are in vitro responses of cultured cells to the presence of lymphocytes, anatomical evidence, and finally, autoradiographic studies on the reutilization of lymphocyte DNA label by other cells in the body.

The in vitro studies of Carrel (1922) and Carrel and Ebeling (1923) established the concept of lymphocyte trophocytes. These workers showed that chicken fibroblasts did not thrive when cultured in serum, but showed pronounced/

pronounced mitotic proliferation when leucocytes or leucocyte extracts were added to the culture. They concluded that leucocytes secreted trephones which may be used as food material by the fibroblasts. More recently, Metcalf (1968) found that thymic or lymph node lymphoid cells would potentiate bone marrow colony growth in vitro and he concluded that lymphocytes can exert trephocytic effects on proliferating haemopoietic cells. The results of other tissue culture studies have shown that the growth promoting ability of lymphocytes may be due to the provision of nucleoproteins. Borenfreund and Bendich (1961) demonstrated the incorporation of polymerized DNA from human leucocytes into the DNA of growing Hela cells. The fact that cultured cells seem to utilize polymerized DNA or DNA complexed to protein rather than soluble DNA (Bensch and King, 1961; Kay, 1961) would appear to add weight to the hypothesis that lymphocytes function as a source of intact nucleoprotein.

Less substantial evidence for the trephocytic hypothesis has come from anatomical studies of the association of lymphocytes with growing or regenerating tissues. Thus, Jordan and Speidel (1923b) believed that the rapid growth of limbs in thyroid-treated tadpoles was due to the stimulating effects of lymphocytes which accumulated there. Similarly, Liebman (1945) demonstrated that lymphocytes are added to the substance of the growing amphibian egg, thus, playing in a sense the role of trephocytes. Experimental observations in mammals have shown that there appears to be a correlation between lymphocyte destruction and lymphopoiesis in germinal centres of lymph nodes (De Bruyn, 1945) and in the thymus (Metcalf, 1966). Finally, a trephocytic function for lymphocytes in haematopoiesis has been suggested by Husse (1963) who correlated the retention/

retention of lymphocytes in the bone marrow with the recovery of erythropoietic and myelopoietic cells following irradiation. He concluded that the results did not support the hypothesis that the lymphocytes acted as multipotential stem cells, but rather, suggested that they were trephocytes.

Of all the evidence that has been presented to support the trephocyte theory, perhaps the most convincing has been that derived from studies on the fate of radioactively labelled lymphocytes. The concept of reutilization of lymphocyte DNA in the production of new lymphocytes has been accepted since the studies of Hamilton (1956), but more recent studies have suggested that lymphocytic nucleic acid may also be available to other cell types. Thus, nuclear labelling following injection of labelled lymphocytes has been demonstrated in ascites tumour cells (Rieke, 1962), bone marrow (Perry, Craddock, Paul and Lawrence, 1959) and regenerating liver (Bryant, 1962). Also, fibroblasts of healing skin wounds become labelled following local infiltration of radioactively labelled lymphocytes (Fichtelius and Diderholm, 1961). It is on the basis of these observations that a trephocytic function of lymphocytes is gradually being accepted, but under the more modern concept of reutilization of nucleoproteins.

The concept of a trephocytic function for lymphocytes in the intestinal epithelium has been strongly upheld in the past (Kelsall and Crabb, 1958). However, in spite of the relatively plentiful evidence suggesting such a function for lymphocytes in other situations, specific evidence relating to those in the intestinal epithelium is singularly lacking or confused. The recent studies of Shields and his colleagues (Shields, /

(Shields, Touchon and Dickson, 1969) suggest that the lymphocytes are intracellular and that more than half of them are degenerating, data which are consistent with a trephocyte hypothesis. These observations, and similar ones by earlier workers (Andrew and Andrew, 1945) represent at the present time the only evidence which can be cited in favour of a trephocyte hypothesis for lymphocytes in the intestinal epithelium. Against this there is evidence that the lymphocytes are not degenerating, but are intercellular and able to migrate back into the lamina propria (Meader and Landers, 1967).

In conclusion, there is a considerable body of evidence suggesting that lymphocytes may function as trephocytes in various situations. The apparent intracellular association of lymphocytes with the intestinal epithelial cells, and their reported degeneration therein suggests that they may function as trephocytes to the absorptive cells.

Nutritional Function.

In contrast with the hypothesis of a trephocytic function for lymphocytes associated with gut epithelium, several authors have postulated that lymphocytes remove nutrients from the epithelial cells and thus act as trephocytes for the rest of the body.

Thus, von Davydoff (1887) suggested that the epithelial cells absorbed nutrients from the lumen of the intestine, and then, by a process of amitotic division, produced the darkly staining lymphocyte which migrated into the lymph and blood. Other authors have also suggested that the lymphocytes may play a part in the absorption, assimilation and transportation of food (Emerson, 1913; Jordan and Speidel, 1923b).

Oppel/

Oppel (1899) put forward the hypothesis that the epithelial leucocytes had the property of carrying out chemical transformations on substances absorbed by the epithelial cells. Zawarykin (1883), and Schafer (1885) advocated a role for lymphocytes in fat absorption. They suggested that the lymphocytes became laden with fat in the epithelium and then migrated back into the tissue of the villus where they discharged it into the lacteals. However, the relative lack of phagocytic activity by lymphocytes would appear not to support this hypothesis.

Although the aforementioned authors suggested a direct nutritional role for lymphocytes, other workers have shown that the nutritional status of an animal appears to affect the gastrointestinal lymphoid tissue in general. Thus, starved animals appear to have reduced gastrointestinal lymphoid tissue activity (Kelsall and Crabb, 1958; Shields, 1968), whereas, there is an increase during digestion (Hofmeister, 1885). This increase appears to be associated with the calorie content of the diet, but more specifically with the fat content, since a high fat diet produces a marked enlargement of the intestinal lymphoid tissue (Settles, 1920 - 1921; Lefholz, 1923 - 1924).

To summarize, several authors have suggested that the lymphocytes in the intestinal epithelium play a role in absorption. Although there is no direct evidence to support this, there is evidence that gastrointestinal lymphoid tissues, such as Peyer's patches and solitary follicles, are affected by the nutritional status of the animal.

CONCLUSION.

It is clear that there is still a great deal of contradictory evidence regarding even relatively 'basic' studies such as morphology, position in the epithelium and rates of labelling with nucleic acid precursors, and that the various hypotheses have little firm support. It is therefore my intention to re-examine certain of the more fundamental characters of these cells such as morphology, and also to examine some of the factors which may affect their numbers, for instance, age, diet, germ-free life, response to cortisone and contact sensitivity. It is hoped that the results may give a more precise indication of the function of these cells, and so help to dispel the present confusion which is apparent in the literature.

CHAPTER 2.A. LIGHT MICROSCOPE STUDIES.INTRODUCTION.

Numerous light microscope studies have been reported on the lymphoid cells in the villus epithelium (von Arstein, 1867; von Davydoff, 1887; Heidenhain, 1888; Hardy and Westbrook, 1895; Beguin, 1904; Guieysse-Pellissier, 1912; Hellman, 1934; Stenqvist, 1934; Blake, 1936; Wolfe-Heidegger, 1939; Andrew and Andrew, 1945; Andrew and Collings, 1946; Kelsall, 1946; Andrew and Sosa, 1947; Kelsall and Crabb, 1958; Andrew, 1965; Meader and Landers, 1967; Shields, Touchon and Dickson, 1969). Unfortunately, many of these reports were chiefly concerned with the authors' hypotheses on the function of these cells and the amount of factual information included was rather scant. However, a certain amount of information has accumulated concerning the morphology of the cells, their position and distribution in the epithelium, and their fate.

The nuclear shape varies from spherical to amoeboid (Wolfe-Heidegger, 1939; Kelsall, 1946), nuclear size is variable (Kelsall and Crabb, 1958), and the nuclear staining indicates that both pachychromatic and leptochromatic nuclei are present (Hardy and Westbrook, 1895). There appears to be little doubt that the majority of the lymphocytes are situated close to the basement membrane (Kelsall, 1946; Kelsall and Crabb, 1958; Andrew, 1965; Darlington and Rogers, 1966; Meader and Landers, 1967; Shields, Touchon and Dickson, 1969), but there have been conflicting reports as to whether or not the lymphocytes are situated intracellularly/

intracellularly (Andrew and Sosa, 1947; Kelsall and Crabb, 1958; Andrew, 1965; Shields, Touchon and Dickson, 1969), or intercellularly (Wolfe-Heidegger, 1939; Andrew and Collings, 1946; Meader and Landers, 1967). There is very little information regarding the distribution of lymphocytes along the length of the villus or along the length of the intestine, with the exception that distribution appears to be irregular (Kelsall and Crabb, 1946), and that there seem to be more lymphocytes in the middle and apical portions of the villus than at the base (Shields, Touchon and Dickson, 1969).

Although the fate of the lymphocytes is difficult to determine from static studies using the light microscope, it has been investigated both in terms of degeneration and migration. The percentage of degenerating cells has been stated to be both low (Kelsall, 1946; Andrew, 1965) and up to 60 per cent (Shields, Touchon and Dickson, 1969). Similarly, some earlier authors suggest that the lymphocytes return to the lamina propria (Zawarykin, 1883; von Davydoff, 1887), others have more recently concluded that they migrate into the lumen of the intestine (Macklin and Macklin, 1932).

It was the purpose of this study to re-examine some of the morphological and distributional features of the lymphocyte population in the epithelium of the small intestine in an attempt to shed more light on its fate and function.

MATERIALS AND METHODS.

Nuclear Diameter.

Five/

Five μm sections were examined using the oil-immersion objective and an eye piece scale with 0.25 μm graduations at this magnification. Only lymphocyte nuclei which were more or less spherical in cross-section were measured, and the focus was adjusted to obtain the maximum diameter. Traversing the field up and down adjacent villi, 100 consecutive round nuclei were measured in each of 5 sections giving a total of 500 measurements.

Degenerative Changes.

Degenerative changes were assessed in terms of nuclear pyknosis. Pyknotic nuclei were defined as intensely and homogeneously stained nuclei with a distinct and smooth border. The pyknotic nuclei in the germinal centres of the Peyer's patches were taken as the reference source with which the nuclei in the epithelium were compared. One thousand nuclei were examined in sections from 10 different mice and the percentage of pyknotic nuclei determined.

Cytoplasmic Pyroninophilia.

Five μm sections of proximal small intestine were stained with methyl green-pyronin, and the lymphocytes in the villus epithelium were examined for cytoplasmic pyroninophilia.

Position Relative to the Epithelial Cell Nucleus.

The position of the lymphoid cell nucleus in the epithelium could be determined by relating it to the epithelial cell nucleus. Three almost equidistant zones could be identified, subnuclear (between the epithelial/

epithelial cell nucleus and the basement membrane), nuclear (level with the epithelial cell nucleus), and supranuclear (between the epithelial cell nucleus and the brush-border). Five hundred nuclei in 1 section from each of 6 animals were classified according to their position. Borderline nuclei were assigned to the position which contained the largest proportion of the nucleus.

Epithelial Cell-Lymphocyte Ratio along the Length of the Villus.

The length of an optimally sectioned villus was approximately $4\frac{1}{2}$ - $5\frac{1}{2}$ fields when determined by a square field eye piece and using an oil-immersion objective. The number of lymphocytes and epithelial cells was determined in 3 zones of the villus, basal, central and apical, each zone being approximately $1\frac{1}{2}$ - $1\frac{3}{4}$ fields in length. For each zone the lymphocyte-epithelial cell index was calculated as $\frac{\text{Lymphocytes}}{\text{Epithelial Cells}} \times 100$. Zone counts were made on 5 villi in sections from 5 different animals.

Epithelial Cell-Lymphocyte Ratio along the Length of the Small Intestine.

A piece of intestine approximately 2-3 cm long was taken from each end, and from $\frac{1}{4}$, $\frac{1}{2}$ and $\frac{3}{4}$ of the way along the length of the small intestine. The number of lymphocytes per 1000 epithelial cells was determined for each of 3 sections from the 5 segments of intestine taken from each of 10 adult mice.

RESULTS.

Nuclear Diameter.

The/

The distribution of the nuclear diameters of 500 lymphoid cells is shown as a histogram in Figure 2.1. The range of the nuclear diameters measured was $3.0\ \mu\text{m}$ - $8.5\ \mu\text{m}$, the majority of nuclei falling in the range $4.0\ \mu\text{m}$ - $6.0\ \mu\text{m}$. However, the frequencies shown do not represent the true frequencies for two reasons. Since spherical nuclei only were measured, there is a bias in the selection of the nuclei, and although taking measurement of 100 consecutive round nuclei rather than 'random' selections tends to reduce the bias, there is no real way of telling whether or not there is any correlation between the size of a nucleus and the probability that it will be spherical. Secondly, there is the technical error in measuring nuclear diameters in sections (Abercrombie, 1946). The probability that the maximum nuclear diameter will be included in a section of given thickness is determined by section thickness divided by the product of section thickness \times nuclear diameter. In other words, the larger the nucleus, the lower the probability that its maximum diameter will be included in the section. Because of this relationship, the histogram in Figure 2.1 is biased to the left. Theoretically, this bias can be corrected for using a relatively complicated formula and a computer (Cormack, 1969). However, a qualitative assessment of the results indicates that small and medium lymphoid cells predominate, the larger lymphoid cells being less frequent.

Degenerative Changes.

The number of pyknotic nuclei per 1000 lymphocytes from 10 different mice was 5; 14; 8; 3; 6; 6; 11; 5; 9; 6 respectively, with a mean figure for degenerating cells of 0.75 per cent.

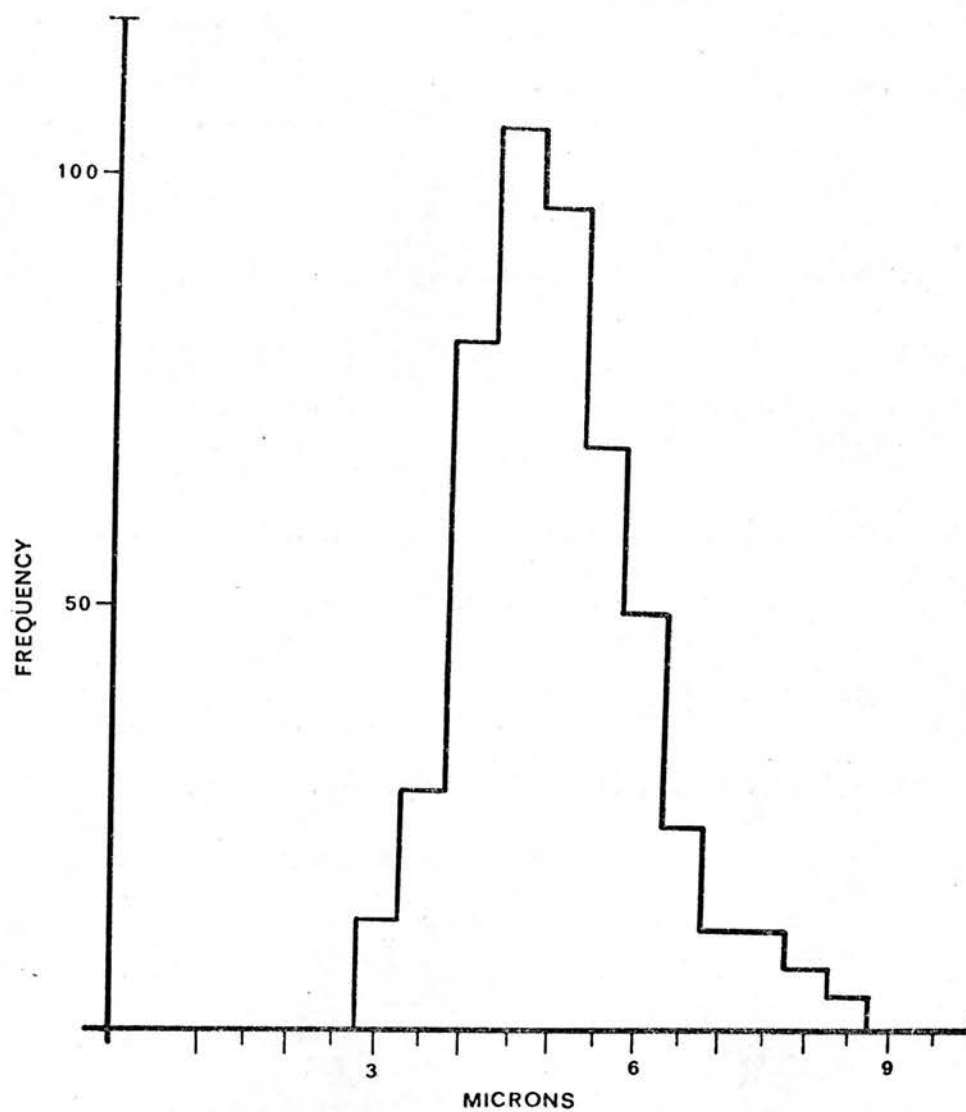


Figure 2.1 Distribution of the nuclear diameters of 200 lymphocytes in sections of the small intestinal epithelium.

Cytoplasmic Pyroninophilia.

The majority of the lymphocytes in the epithelium did not have pyroninophilic cytoplasm, nor were pyroninophilic nucleoli prominent. Of the remainder, 3 types of pyroninophilic cell could be identified.

(a) Large Pyroninophils:- These cells had nuclei much larger than those of the adjacent epithelial cells. The nucleus stained lightly with methyl green and there were usually one or more large pyroninophilic nucleoli. The cells were characterized by abundant pyroninophilic and often vacuolated cytoplasm which extended right round the nucleus, so that on tangential section these cells appeared as smaller pale-staining nuclei surrounded by a prominent rim of pyroninophilic cytoplasm. These cells constituted less than 1 per cent of the lymphocytes in the epithelium.

(b) Medium Pyroninophils:- These cells had nuclei of approximately the same diameter as those of the epithelial cells. The nucleus stained more intensely with methyl green compared to the nucleus of the large pyroninophil, and contained smaller and less prominent nucleoli. On optimal sectioning, deeply pyroninophilic cytoplasm, slightly denser than that of the epithelial cells, was present mainly at one pole of the cell, with only a very thin rim around the nucleus. In tangential sections it was very difficult to differentiate these cells from the larger non-pyroninophilic cells in the epithelium. The medium pyroninophils occurred with a greater frequency than the large pyroninophils, but it was difficult to quantitate them because of the problem of tangential sectioning, and because of the small difference in the staining intensity of their cytoplasm and that of the surrounding epithelial/

epithelial cells. However, counting definite positive pyroninophils in sections from 25 mice indicated that approximately 5 per cent of the lymphoid cells in the epithelium had pyroninophilic cytoplasm (5000 cells scored; Mean 5.4 per cent; Range 2.5 per cent - 11.0 per cent).

(c) Plasma Cells:- Cells with the classical morphology of plasma cells were found in the epithelium only very infrequently and no attempt was made to quantitate them.

Position Relative to the Epithelial Cell Nucleus.

Of the 3000 nuclei examined, 83.5 per cent were subnuclear, 15.3 per cent were nuclear and only 1.2 per cent were supranuclear (Table 2.1).

TABLE 2.1.

Distribution of lymphocytes relative to the position of the epithelial cell nucleus.

Position	Mouse Number						Total	Per Cent
	1	2	3	4	5	6		
Subnuclear	421	394	430	438	417	404	2504	83.5
Nuclear	73	101	61	57	77	89	458	15.3
Supranuclear	6	5	9	5	6	7	38	1.2

Epithelial Cell-Lymphocyte Ratio along the Length of the Villus.

The distribution of lymphocytes along the length of the villi is presented in Table 2.2. Analysis of the ratio of lymphocytes to epithelial cells in the basal, central and apical portions of the villi indicated/

TABLE 2.2.

Distribution of lymphocyte: epithelial cell index ($\frac{L}{E} \times 100$)
in the basal, central and apical portions of the villus.

Mouse Number	Villus Number	Lymphocyte: Epithelial Cell Index		
		Basal	Central	Apical
1	1	14.7	20.3	11.3
	2	8.9	18.5	17.7
	3	5.8	22.1	19.7
	4	17.5	23.7	23.3
	5	12.1	33.3	21.2
2	1	10.9	19.4	14.9
	2	7.0	15.2	6.0
	3	8.6	25.6	21.6
	4	7.2	18.1	20.8
	5	18.6	32.3	23.2
3	1	17.3	47.7	24.6
	2	20.5	41.3	29.4
	3	16.4	43.0	23.5
	4	14.3	24.3	32.8
	5	8.4	43.9	26.7
4	1	18.1	32.6	35.9
	2	13.9	28.8	24.2
	3	17.9	26.2	16.7
	4	12.7	22.9	16.2
	5	18.9	25.4	29.2
5	1	6.5	12.0	5.3
	2	8.5	6.3	7.8
	3	5.7	17.8	6.4
	4	5.4	8.0	5.0
	5	6.0	10.8	7.6
Mean \pm S.D.		12.1 \pm 5.1	24.8 \pm 11.1	18.8 \pm 8.9

indicated that there was a definite zonal distribution. The basal portion of the villus contained the lowest number of lymphocytes, the central portion the highest, with the apical portion occupying an intermediate position. These zonal distributions were significantly different (P 0.01 - 0.05).

Epithelial Cell-Lymphocyte Ratio along the Length of the Small Intestine.

TABLE 2.3.

Number of lymphocytes per 1000 epithelial cells in segments of small intestine from the proximal (1) and distal (5) regions and $\frac{1}{4}$ (2), $\frac{1}{2}$ (3) and $\frac{3}{4}$ (4) of the way along its length. Each figure is the mean of 3 counts.

Mouse Number	Number of lymphocytes per 1000 epithelial cells in regions				
	1	2	3	4	5
1	23	28	33	37	23
2	39	41	32	24	25
3	21	35	41	40	24
4	32	28	28	34	35
5	39	34	24	36	33
6	25	23	20	25	33
7	20	22	18	28	25
8	35	29	26	20	21
9	46	44	30	57	70
10	26	20	17	19	30
Mean \pm S.D.	30.6 \pm 8.9	30.4 \pm 8.0	26.9 \pm 7.5	32.0 \pm 11.4	31.9 \pm 14.2

The results presented in Table 2.3 indicate that in the 10 mice studied/

studied there were only minor variations in the number of lymphocytes per 1000 epithelial cells in different regions of the intestine.

DISCUSSION.

Light microscopic studies of the lymphocyte population in the villus epithelium indicate that this population is heterogeneous in morphology. The nuclear diameter on section ranges from $3.5\text{ }\mu\text{m}$ - $8.0\text{ }\mu\text{m}$ (mode $4.5\text{ }\mu\text{m}$ - $5.0\text{ }\mu\text{m}$), which is in close agreement with the figures reported by Kelsall (1946) for the hamster. The nuclei also vary from pachychromatic to leptochromatic in character as described by Hardy and Westbrook (1895). The larger nuclei have the more open chromatin patterns. Cytoplasmic pyroninophilia has been described in non-mammalian species and this is especially prominent in the frog (Fichtelius, Finstad and Good, 1969). These results show that it is also a feature of some of the lymphoid cells in the mouse.

In general, it can be said that the range of lymphoid cell morphology seen in the villus epithelium is as great as that found in the lymphoid organs elsewhere in the body. Since small lymphocytes can be induced to transform into a variety of morphological forms in vivo and in vitro (Gowans, McGregor, Cowen and Ford, 1962; Porter and Cooper, 1962; Ling, 1968), the morphological heterogeneity found in the cells in the epithelium raises the question of whether or not these cell types represent the morphological variations of one population, or whether they represent two or more distinct populations. Unfortunately, this point cannot be resolved using light microscopy alone.

An/

An indication of the fate and function of these lymphocytes can be gained by analysing their morphology and distribution. Part of the morphological variation seen in the lymphoid cells is manifested as degeneration. If the term degeneration is limited to cells which have pyknotic or karyorrhectic nuclei, then less than 1 per cent of the cells are degenerating, which is in agreement with the results of Kelsall (1946) and Andrew (1965). This low level of degeneration, and the scarcity of DNA fragments found using the Feulgen reaction (Kelsall and Crabb, 1958), does not support the classical trephocyte or reutilisation hypothesis which suggests that lymphocyte DNA trephones are made available to other dividing cells by lymphocyte degeneration. This hypothesis is exemplified histologically by the cell degeneration found in the thymus (Metcalf, 1966; Claesson, 1969) and in the germinal centres (Fliedner, Kesse, Cronkite and Robertson, 1961; Swartzendruber and Congdon, 1963).

If very few of these cells die, then what is their ultimate fate? The distribution of the lymphocytes in the epithelium provides some indication. The virtual restriction to the nuclear and subnuclear regions of the epithelium (Kelsall, 1946; Darlington and Rogers, 1966), and the failure to observe any cells actually in the process of crossing the brush-border, suggests that the lymphocytes do not migrate into the lumen whilst they are in the epithelium covering the sides of the villi. If they do migrate through into the lumen then they must spend a minute fraction of their transit time crossing the supranuclear portion of the epithelium, and this is the very region where there are complex interdigitations and various types of junction between adjacent epithelial cell membranes (Toner, 1968). However, this situation does not hold at the/

the extrusion zone at the tip of the villus, and therefore it is probable that lymphocytes are shed or even actively migrate into the lumen from this zone, although none were actually seen.

A more definite indication of the population changes in the apical regions of the villi can be obtained when the distribution of lymphocytes along the length of the villus is considered. The largest number of lymphocytes is found in the central portion of the villus, followed by the apical portion, the smallest number being found in the basal portion. There are two possible explanations of this distribution. The first assumes that the lymphocyte migration streams are independent of the epithelial cell migration from the base of the villus to the tip, that is, the lymphocytes in the epithelium can migrate with or against the epithelial cell stream so that there is a net pooling of cells towards the middle of the villus. Alternatively, it can be assumed that lymphocytes are more or less restricted to the intercellular space into which they migrate and that any further migration is either into or out of the epithelium and not in a direction parallel to the basement membrane. In this case, the ratio of lymphocytes to epithelial cells will remain constant in a particular zone as this zone is carried towards the tip of the villus, unless there is net addition to, or removal of cells from the zone.

Applying this latter concept to the distribution found in the villi, then in the basal and central zones the net migration is into the epithelium, whereas, the decrease in ratio from central to apical zones indicates that in the apical zone there is a net loss from the epithelium. Since it is unlikely that this loss is into the lumen of the intestine, except/

except possibly at the very tip of the villus, then some lymphocytes must migrate back into the lamina propria. Although there is no evidence to suggest that lymphocytes cannot migrate actively in a direction parallel to the basement membrane and thus show a net pooling in the centre of the villus, it is considered more likely that lymphocytes are carried passively towards the tip of the villus as the epithelial cell - basement membrane complex moves along (Pascal, Kaye and Lane, 1968), and that at least a proportion of the cells which migrate into the epithelium in the basal and central zones of the villus return to the lamina propria. Although it is not possible to determine the magnitude of migration into the lamina propria from the apical zone of epithelium, the presence of a reduced, but still relatively substantial 'residual' lymphocyte population, suggests that a proportion of the cells may remain in the epithelium to be shed into the lumen of the intestine at the tip of the villus.

Finally, we have to consider the distribution of lymphocytes along the length of the small intestine. Although the distribution of lymphocytes along the length of the villus is variable, the distribution of these cells along the length of the intestine is relatively uniform when expressed in terms of lymphocytes per 1000 epithelial cells. However, since the size of the villi diminishes distally along the small intestine, then, in absolute numbers, the number of lymphocytes in the epithelium also decreases distally.

This distribution of lymphocytes lends itself to two possible interpretations. Because of the constant ratio of lymphocytes to epithelial cells along the length of the intestine of any one animal, the lymphocytes may be associated with epithelial cell maintenance and function/

function as suggested by the trephocyte hypothesis (Shields, Touchon and Dickson, 1969), the differences between animals reflecting differing functional demands by the epithelial cells. Alternatively, the constant ratio may be purely fortuitous with larger numbers of lymphocytes migrating to the proximal small intestine, which coincidentally has a greater epithelial area. In this latter case the lymphocytes will be of greater functional significance in the anterior rather than the posterior small intestine. In the absence of any specific evidence, further discussion of the function of these cells is unwarranted except to emphasize the two possibilities suggested by their distribution, namely that, in terms of the epithelial cells, they have a relatively constant proportion along the length of the intestine, but that by virtue of this relationship, the lymphocytes are more numerous in the anterior intestine.

To sum up, the lymphocytes in the villus epithelium have a heterogeneous morphology. Their fate is also variable, a few degenerate, some return to the lamina propria, and others may be shed into the lumen of the intestine. Their function is unknown, but they are numerically superior in the anterior small intestine and they are present in a relatively constant ratio with the epithelial cells along the length of the small intestine.

B. FLUORESCENCE MICROSCOPE STUDIES.

INTRODUCTION.

Hellman (1934) was one of the first proponents of the hypothesis that/

that lymphocytes in the intestinal epithelium are part of the defensive barrier of the intestine. If one interprets this concept of defence in terms of the known functions of lymphoid tissue, then it is possible that the cells in the intestinal epithelium function as effector cells in either a humoral defence mechanism or in a cellular one. The presence of pyroninophilic cells in the epithelium, and the finding by Whur and White (1970) that cells in the epithelium with the morphology of small lymphocytes fluoresce when stained with specific antiserum, suggests that the function of these cells could be that of antibody production.

The concept of a local immune mechanism has been concerned mainly with secretory immunoglobulins. Many epithelial surfaces have lymphoid cells associated with them, and, especially in the intestine, a large number of these cells are pyroninophilic. Staining with specific fluorescein-conjugated antisera has shown that a large number of lymphoid cells associated with epithelial surfaces contain IgA. In the intestine the majority of these cells are situated in the lamina propria near the base of the villi. This corresponds with the greatest concentration of pyroninophilic cells. The prominence of IgA containing cells over the number containing IgG and IgM, indicates that the epithelial-associated lymphoid tissue must be considered to be somewhat different from that situated deeper in the body where IgG containing cells predominate (Crabbe, Carbonara and Heremans, 1965; Brus, Siegel and Glass, 1967; Gelzayd, Kraft and Kirsner, 1968; Heremans, 1968; Tomasi and Bienenstock, 1968; Martinez-Tello, Braun and Blanc, 1969; Nash, Vaerman, Bazin and Heremans, 1969; Smith, 1969).

Not only do cells in the intestinal mucosa contain predominantly IgA/

IgA, but the predominant antibody in the intestinal fluid is IgA (Plaut and Keonil, 1969). Thus, there is little doubt that antibodies produced in the intestinal mucosa gain access to the intestinal lumen. However, there is still some discussion as to the mechanism of this secretion. There is evidence that proteins can reach the intestinal lumen via the interstitial fluid and there does not appear to be any molecular filtration mechanism since numerous proteins can pass easily into the gut (D'Addabbo, Dammacco, Schena and Dalfino, 1968). Heremans and Crabbe (1967) have suggested that transfer of IgA from the lamina propria to the intestinal lumen also occurs by passive seepage through intercellular junctions. However, exocrine IgA has an additional complex attached to it, which has been termed 'secretory piece' (Tourville, Adler, Bienenstock and Tomasi, 1969). This substance is not secreted by the plasma cells in the lamina propria, but appears to be attached to the immunoglobulin molecule in the intestinal epithelium and hence it has been postulated that this is associated with the transport of IgA into the intestinal lumen.

The presence of pyroninophils amongst the lymphoid cells in the intestinal epithelium suggests that they may also be involved in the secretion of antibodies in addition to the pyroninophils in the lamina propria. The non-pyroninophilic cells in the epithelium may also be involved in antibody production since recent investigations have shown antibody in association with various types of lymphocytic cell. Thus, antibody determinants have been demonstrated in, or on the surface of lymphocytes by fluorescent antibody techniques (Lobb, 1968; van Furth, 1969; Raft, Sternberg and Taylor, 1970); by immunocytoadherence (Storb, Chambers, Storb and Weiser, 1967; Storb and Weiser, 1967; McConnell, Munro/

also
refs.

Munro, Gurner and Coombs, 1969; Storb, Bauer, Storb, Fliedner and Weiser, 1969); by microdrop studies (Atardi, Cohn, Horibata and Lennox, 1964); by agar plaque techniques (Bussard and Binet, 1965; Harris, Hummeler and Harris, 1966; Shearer, Cudcowicz, Connell and Priore, 1968); by reacting lymphocytes with antisera directed against specific immunoglobulin determinants (Wilson, Munro and Coombs, 1969; Dwyer and Mackay, 1970; Mason and Warner, 1970; Warner, Byrt and Ada, 1970); by electron microscope autoradiography (Bosman, Feldman and Pick, 1969; Mandel, Byrt and Ada, 1969) and by immunochemical methods (Bert, Massaro and Maja, 1968; Chessin, Glade, Asofsky, Baker, Reisfeld and Terry, 1968; Merler and Janeway, 1968; Bert, Massaro, Di Cossano and Maja, 1969). If similar cells are present in the intestinal epithelium, this may account for the finding by Whur and White (1970) that some cells, with the morphology of small lymphocytes, show specific fluorescence when stained with conjugated antisera.

Therefore, it was the purpose of this study to use the fluorescent antibody technique to test the hypothesis that antibody secretion is a major function of the lymphoid cell population in the intestinal epithelium.

MATERIALS AND METHODS.

The details of the techniques used are presented in the Appendix (p. 243). Cryostat sections of intestine, 6 μ m - 8 μ m thickness, were fixed in methanol for 10 minutes and then stained with rabbit anti-mouse immunoglobulin for 30 minutes. The sections were rinsed/

rinsed in phosphate buffered saline and stained for 30 minutes with fluorescein-conjugated goat anti-rabbit IgG (Miles Laboratories). Stained sections were rinsed in phosphate buffered saline, mounted in glycerol, and examined on a Zeiss fluorescence microscope. Control sections were stained directly with the conjugate or indirectly after prior incubation with rabbit anti-Newcastle disease virus serum.

RESULTS.

Specific fluorescence was found mainly in the cells in the lamina propria of the lower two-thirds of the villus (Plates 2.2 and 2.3). Only occasional cells in the intestinal epithelium were observed to fluoresce (Plates 2.4 and 2.5). In the case of the fluorescing cells in the epithelium, the fluorescence was usually located at one pole of the cell, a location analogous with the pyroninophilic cytoplasm of some of the cells stained by methyl green-pyronin and examined by ordinary light microscopy (p. 24).

DISCUSSION.

Although a few cells in the epithelium showed specific fluorescence with conjugated antisera against immunoglobulin determinants, these cells were in the minority. The results agree with those of Whur (1970) who found that although small lymphocytes in the epithelium did fluoresce, there were only occasional cells in each villus which did so.

The/

The relative absence of fluorescing cells in the epithelium could be due to the sensitivity of the technique. Since it has been well established that lymphocytes have immunoglobulin determinants on their surface (Sell and Asofsky, 1968) then it might be expected that all the lymphocytes in the epithelium would fluoresce. However, with a low concentration of determinants, it would be difficult to detect specific fluorescence above the background fluorescence, and under the present experimental technique specific bright fluorescence was limited to cells containing high levels of antibody such as the plasma cells in the lamina propria. The conclusion drawn from the observation that the epithelium is relatively lacking in fluorescing cells compared to the lamina propria, is that antibody production is not a major function of the lymphocyte population in the intestinal epithelium.

Plate 2.1 Control section of small intestine stained with rabbit anti-Newcastle disease globulin and then with fluorescein-conjugated goat anti-rabbit IgG (diluted 1/10).
x 1000 (approx.).

Plate 2.2 Specific fluorescence of cells
and in the lamina propria of small
Plate 2.3 intestine stained with rabbit anti-mouse immunoglobulin and then with fluorescein-conjugated goat anti-rabbit IgG (diluted 1/10). These are sections of a similar area to that in Plate 2.1 with the epithelium situated in the upper right portion of the photograph. Note the dull granular fluorescence in some of the cells in the lamina propria and the bright ring of fluorescent cytoplasm of neighbouring cells.
x 1000 (approx.).

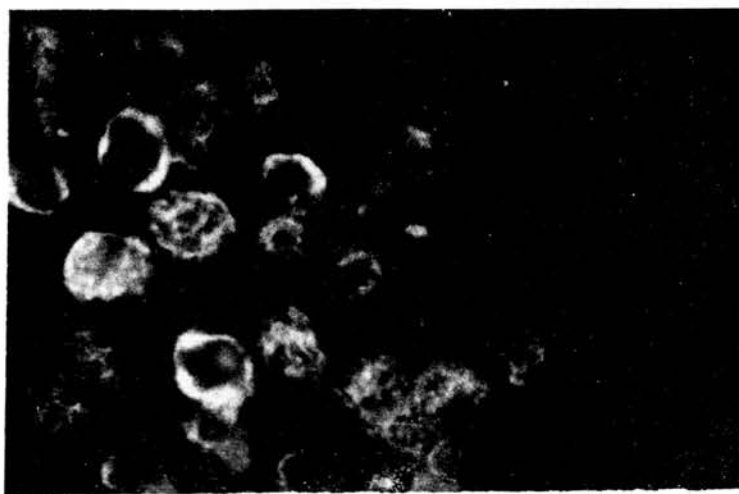
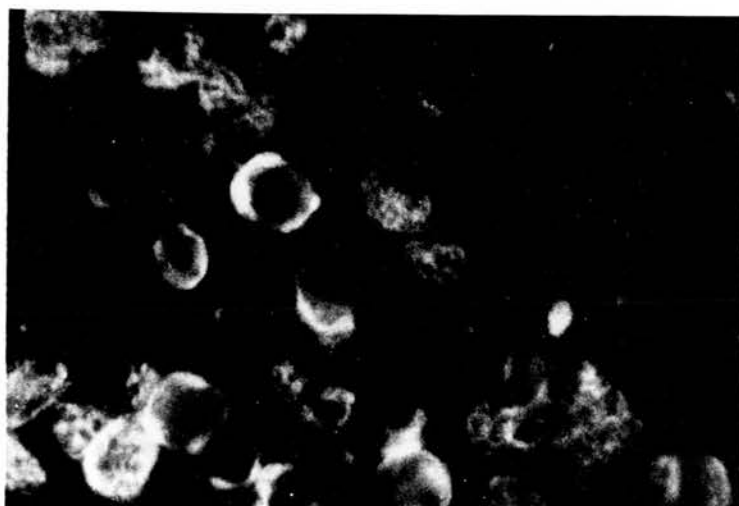
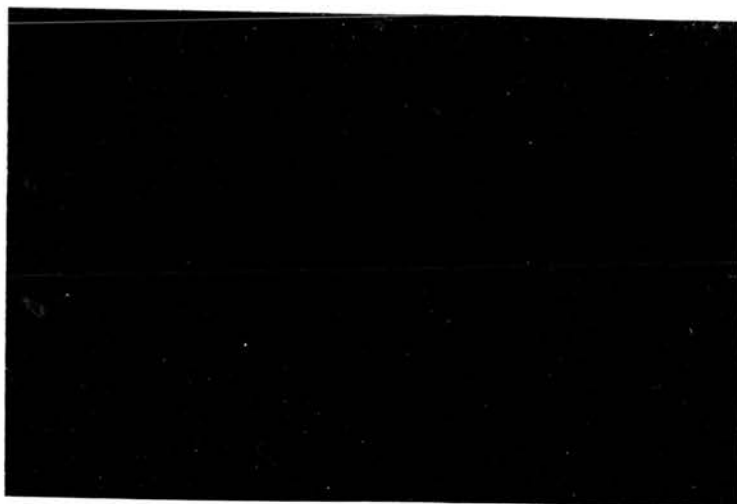
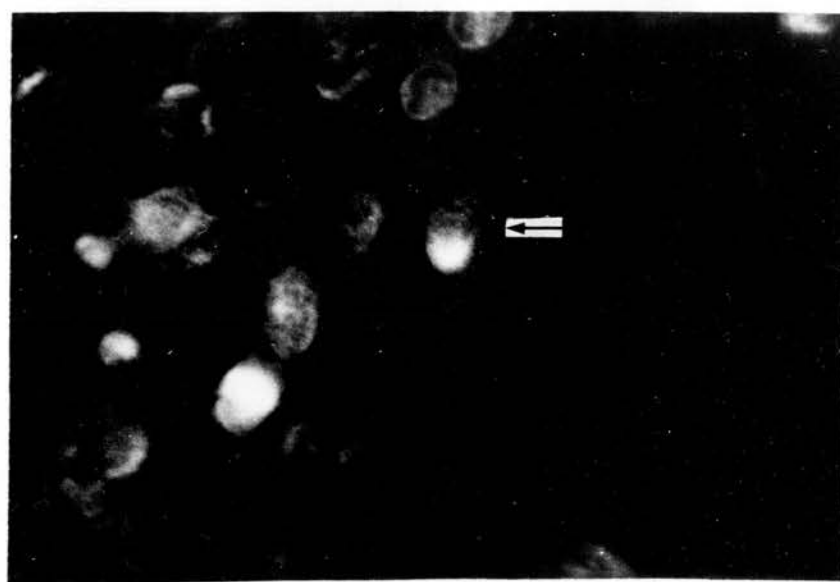
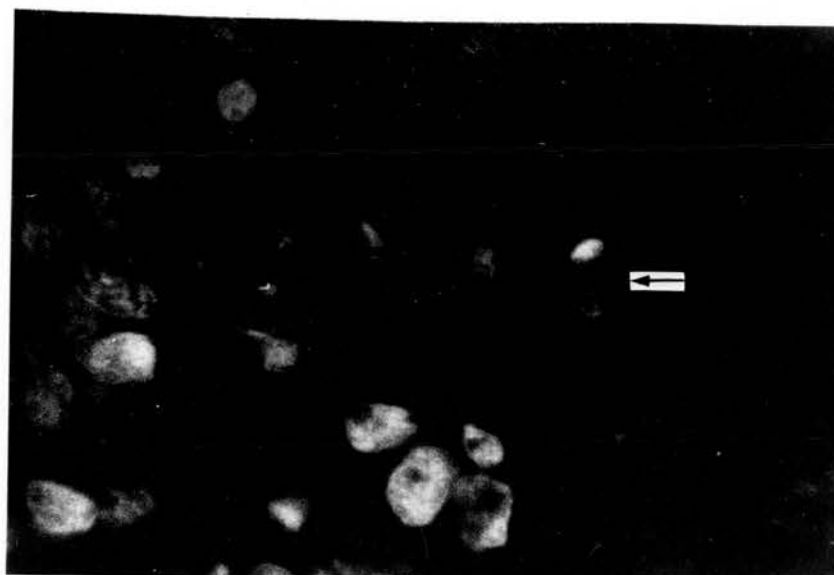


Plate 2.4 Section of small intestine stained with rabbit anti-mouse immunoglobulin and then with fluorescein-conjugated goat anti-rabbit IgG (diluted 1/16). Note specific fluorescence of the cytoplasm at one pole of a cell situated in the epithelium (arrow).
x 1000 (approx.).

Plate 2.5 Section of small intestine stained with rabbit anti-mouse immunoglobulin and then with fluorescein-conjugated goat anti-rabbit IgG (diluted 1/10). Note specific fluorescence of the cytoplasm at one pole of a cell situated in the epithelium (arrow). A brightly fluorescing cell is also present in the lamina propria.
x 1000 (approx.).



CHAPTER 3.ELECTRON MICROSCOPE STUDIES.INTRODUCTION.

Electron microscope studies on lymphocytes in the epithelium of the small intestine have been reported by Palay and Karlin (1959), Andrew (1965) and Meader and Landers (1967). Palay and Karlin were primarily concerned with the epithelial cells, but commented on the presence of 'intrusive' small macrophage-like cells migrating into the epithelial layer between the epithelial cells. Andrew (1965) and Meader and Landers (1967) reported the results of more comprehensive studies, but there is little agreement on either the morphology of the lymphocytes or their precise relationship to the epithelial cells. Andrew noted a peculiar type of transformation in the infiltrating cells, comprising a decrease in the number of mitochondria and a loss of endoplasmic reticulum, and stated that they could not be considered the same as lymphocytes in the lamina propria. On the other hand, Meader and Landers did not confirm such a transformation and concluded that the lymphocytes in the epithelium were morphologically similar to those seen in the lamina propria except that they contained more lysosome-like dense bodies.

On the question of position in the epithelium, Andrew reported that his studies supported the concept of an intracellular position for some of the lymphocytes, whereas Meader and Landers favoured an inter-cellular position. Meader and Landers also examined the problem of lymphocyte migration, and while clearly demonstrating that the lymphocytes migrated into the epithelium from the lamina propria, they further suggested/

suggested that they may also migrate from the epithelium back into the lamina propria.

There is clearly some controversy about the fine structure of the lymphocytes in the epithelium and it was the purpose of this study, therefore, to examine not only the cells themselves but also their relationship both to each other and to the epithelial cells, as well as to obtain evidence of their directions of migration.

MATERIALS AND METHODS.

The details of the techniques used are presented in the Appendix (p. 247). Mice were killed by cervical dislocation. A proximal segment of small intestine was removed and the lumen perfused with 10 per cent formalin. The segment was fixed for approximately 1 hour and then cut into smaller pieces for post-fixation in osmium tetroxide. Following fixation, the specimens were dehydrated and embedded in araldite and thin sections were cut for examination in an AEI EM6B microscope.

RESULTS.

Absorptive epithelial cells with a brush-border, goblet cells secreting mucus, and argentaffin cells with characteristic granules were readily identified. The observations, however, were confined to the lymphoid cells in the epithelial layer. The structure of the lymphoid cells, their relationship to each other and the epithelial cells, and the question of direction of migration were all examined.

Structure.

After examination of several sections, it soon became apparent that the structure of the lymphoid cells was not uniform and eventually it was possible to classify the cells into two distinct groups, lymphocytes and blast cells.

The lymphocytes were the predominant cell type seen, and although a range of morphology was observed, their basic structure was similar. They were round, or more usually, ovoid in shape. The dense nuclear chromatin showed peripheral condensation, and single nucleoli were occasionally seen. There was no indication of nuclear degeneration. The sparse non-granular cytoplasm contained only a few ovoid mitochondria, but occasional cells contained twelve or more. The mitochondria were usually found as a small group close to the nucleus. Occasionally, large, membrane-bound electron-dense bodies $0.3\ \mu\text{m}$ - $0.6\ \mu\text{m}$ in diameter were seen in the cytoplasm (Plates 3.9 and 3.10). Some lymphocytes contained two or three of these bodies. The Golgi apparatus was usually small or absent and a moderate number of free ribosomes were distributed throughout the cytoplasm.

Although the lymphocytes had the basic morphological structure described above, there was quite a wide variation in the size of the cells and also in the number of Golgi-associated cytoplasmic membranes. The majority of cells examined had diameters ranging from $5\ \mu\text{m}$ - $9\ \mu\text{m}$, but occasionally larger cells were encountered with diameters up to $12\ \mu\text{m}$ - $14\ \mu\text{m}$. The other main morphological variable was the quantity of cytoplasmic membranes. In the majority of cells, there were few Golgi lamellae or vesicles, but some cells contained a considerable number of membranes/

membranes and vesicles (Plates 3.3 and 3.4). In general, there was a tendency for the larger cells to have more cytoplasmic membranes, but this correlation was by no means true for all cells examined.

The blast cells seen in the epithelium were two to four times larger than the small lymphocytes, and were only encountered in occasional sections. These cells had large oval nuclei with sparsely clumped chromatin, and one or more prominent nucleoli. The characteristic feature of these cells was the large number of polysomes in the cytoplasm together with a few large, round or oval mitochondria. Electron-dense inclusions up to 1 μ m in diameter were sometimes seen. The Golgi apparatus was moderately well developed, and a few short slender profiles of rough and smooth endoplasmic reticulum were sometimes present (Plates 3.5 and 3.6).

Cell - Cell Relationships.

The majority of the lymphoid cells were situated singly in the epithelium, but occasionally two or more cells were seen in contact with each other. Small lymphocytes were the most common combination (Plate 3.7), but in one instance, a small lymphocyte was observed in contact with a blast cell (Plate 3.8).

Lymphoid - Epithelial Cell Relationship.

The majority of the lymphoid cells were situated close to the basement membrane, but occasional cells were seen between the epithelial cell nuclei and the brush-border. There was no doubt in any of the sections examined that the lymphoid cells were situated between the epithelial cells and not in them. In no case was there any suggestion that/

that the cells were intracellular (Plates 3.1 and 3.2).

Lymphoid Cell Migration.

Cells migrating through the basement membrane were sometimes seen. Since some lymphocytes have a polarized movement (Lewis, 1931), and using as a basis for ultrastructural polarity pseudopodial extension at one pole of the cell with the trailing of cytoplasmic contents at the opposite pole (Meador and Landers, 1967), then lymphocytes were in the process of migration from the lamina propria into the epithelium. This is in accord with the observations of Andrew (1965) and Meador and Landers (1967). In one instance a blast cell was seen migrating into the epithelium (Plate 3.5). Occasionally, a small lymphocyte was encountered which appeared to be migrating from the epithelium into the lamina propria. Examples of this are shown in Plates 3.11 and 3.12 and demonstrate the disruption of basement membrane and collagenous fibres, in the direction of migration, by the advancing pseudopodium, and trailing of cytoplasmic contents towards the rear of the cell. Small lymphocytes were the only cell type seen migrating in this direction.

DISCUSSION.

The structure of the lymphocytes in the intestinal epithelium is similar to that described for lymphocytes in the blood (Bessis and Thiery, 1961), thoracic duct (Zucker-Franklin, 1963; Feldman and Nordquist, 1967; Hebel and Liebich, 1969; Wivel, Mandel and Asofsky, 1970) and in lymph nodes (Sorenson, 1960; Han, 1961; Moe, 1964) of various/

various species. In all these situations, although cells can be classified as typical lymphocytes on accepted cytologic criteria such as condensation of nuclear chromatin, high nuclear-cytoplasmic ratio, small Golgi complexes, small number of mitochondria, and occasional large inclusion bodies (Feldman and Nordquist, 1967), there is a marked heterogeneity within the population (Han, 1961; Zucker-Franklin, 1963). The lymphocytes in the intestinal epithelium show a similar heterogeneity of ultrastructure and size. The lymphocytes could be divided, on the basis of size, into large, medium and small, but there is considerable overlap, as is the case in, for example, lymph nodes (Han, 1961). However, the smaller cells were predominant.

These observations agree with those of Meader and Landers (1967) for lymphocytes in the epithelium, in that the lymphocytes showed no signs of degeneration, and that they were similar to the lymphocytes in the lamina propria except that they contained more lysosome-like dense bodies. The transformation reported by Andrew (1965) was not observed and it is possible that this author was comparing the lymphocytes in the epithelium with the preplasmacytes in the lamina propria, in which case the lymphocytes could be described as demonstrating a marked decrease in the number of mitochondria and in the amount of endoplasmic reticulum.

In contrast to the marked heterogeneity of the lymphocytes in the epithelium, the few blast cells observed had a fairly characteristic and uniform ultrastructure. It is unfortunate that the nomenclature surrounding these cells is not so uniform, and the cells here termed blast cells are considered to be similar to cells described variously as transitional cells (Fragraeus, 1968), large lymphoid cells (Scothorne and/

and McGregor, 1955), haemocytoblasts (Andre, Schwartz, Mitus and Dameshek, 1962), immunoblasts (Dameshek, 1963) and several other names. In all cases the cells concerned are large, have vesicular nuclei with prominent nucleoli, and a cytoplasm characterized by the presence of numerous polyribosomes. These cells have not been described previously as present in the intestinal epithelium, but they have been described in other lymphoid tissues during the initial stages of antibody production (Movat and Fernando, 1965), delayed hypersensitivity (Turk and Heather, 1965), homograft rejection (Binet and Mathe, 1962) and graft versus host reactions (Porter and Cooper, 1962). In the latter reaction, the blast cells appear to be derived from small lymphocytes. The presence of these cells in the intestinal epithelium would therefore suggest that some form of immunological response is taking place here.

The majority of the lymphoid cells were situated singly in the epithelium, but occasionally two or more cells were seen in contact. Small lymphocytes were the cells most frequently seen in contact, as is the case in lymph nodes. Sura and his colleagues described contact between lymphocytes in tissue culture, and suggested that there was transfer of nucleic acid between cells (Sura, Kadaghioze, Slavina and Svet-Moldavsky, 1968). However, the bridge-like fusions between adjacent lymphocytes, as described by Sura and co-workers were not seen in this study. The contact between lymphocytes and blast cells has also been described in immunological reactions in vitro (McFarland, Heilman and Moorhead, 1966). The association between blast cell and lymphocyte seen in this study may represent a similar interaction.

The position of the lymphoid cells in the epithelium is in agreement/

agreement with that reported by Meader and Landers (1967), namely that they are situated close to the basement membrane and are between the epithelial cells. There was no evidence for the lymphocytes being intracellular as described by Andrew (1965). The predominantly basal position of the lymphoid cells, and the failure to observe any cells crossing the brush-border into the lumen of the gut is not in accord with the suggestion that lymphocytes actively migrate into the lumen. However, it is possible that the lymphocytes may enter the lumen of the intestine passively by being shed from the tip of the villus together with the epithelial cells.

Lymphocytes are actively mobile cells and have been said to migrate as fast or even faster than polymorphs in vitro (McCutcheon, 1924; Abramson, 1927). They have a polarized movement (Lewis, 1931), and on the few occasions in which sections of lymphocytes traversing the basement membrane were obtained, it could be deduced (from the criteria outlined above) that lymphocytes migrated both into the epithelium from the lamina propria and vice-versa. It is impossible to determine the magnitude of the migration from the epithelium into the lamina propria in ultrathin sections, so whether such migration is a characteristic of a subpopulation or the whole lymphocyte population is not known. Although small lymphocytes are the only cell type observed migrating from the epithelium to the lamina propria, this may merely be a reflection of the higher probability of encountering this cell type in thin sections, and does not exclude the possibility that the more infrequent larger lymphocytes and blast cells also migrate the same way. Whether cells migrate beyond the lamina propria is not known, but lymphocytes have been described in the lacteals/

lacteals (Palay and Karlin, 1959), and a proportion may thus enter the thoracic duct lymph.

The significance of the return of lymphocytes back into the lamina propria is unknown, but it does suggest that they may be acquiring some nutritional factor or immunological information, and 'delivering' back into the body.

To sum up, electron microscopic studies were made on the non-epithelial cells in the epithelium of the small intestinal villi of adult mice. The results show that this population is heterogeneous, consisting of lymphocytes, of varying size and structure, and large blast cells. The small-medium lymphocytes were the predominant cell type. All the cells examined were situated between the epithelial cells, the majority being close to the basement membrane, through which some small lymphocytes could be seen migrating into the lamina propria. Different cell types were sometimes observed in close contact with each other.

Plate 3.1 The small lymphocyte (LC) is the most common non-absorptive cell type found in the epithelium. It is characterized by relatively scanty cytoplasm containing a few mitochondria and few, if any, Golgi membranes. The intercellular position of this cell can be clearly seen as it lies in a triangular space bounded by three epithelial cells (E). The arrows indicate the complex interdigitations of cell membranes of the epithelial cells where they are in contact. x 7500 (approx.).

Plate 3.2 A small lymphocyte situated similarly as that in Plate 3.1. Chylomicrons are present in the intercellular space (arrow). x 10000 (approx.).

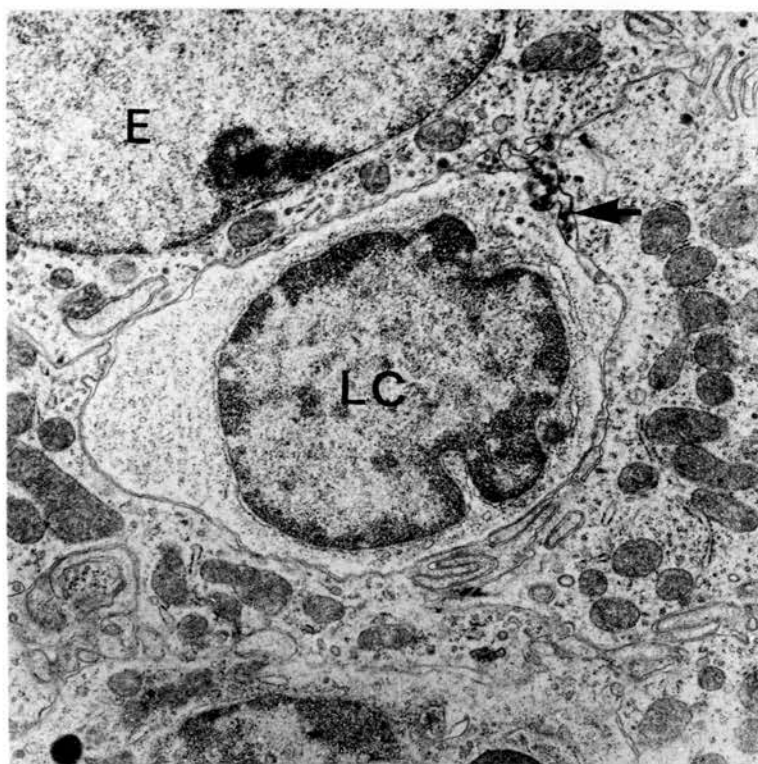
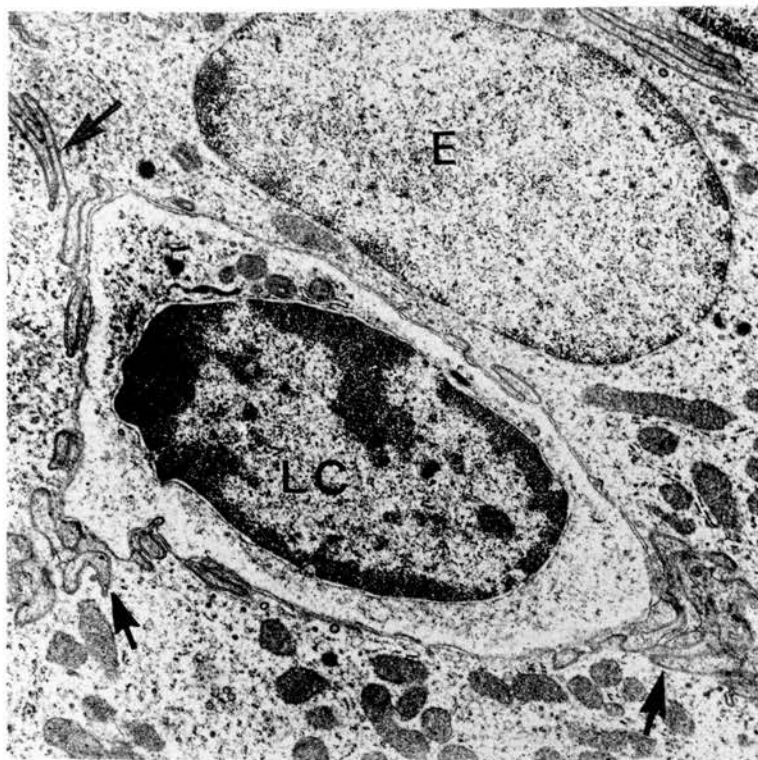


Plate 3.3
and
Plate 3.4

Lymphocytes with a more complex cytoplasmic structure. The mitochondria are more numerous, the Golgi apparatus is well developed and there are numerous small vesicles in the cytoplasm. There is a moderate amount of rough endoplasmic reticulum. Both these cells are situated at the base of the epithelial cells close to the basement membrane (arrows).
x 7500 (approx.).

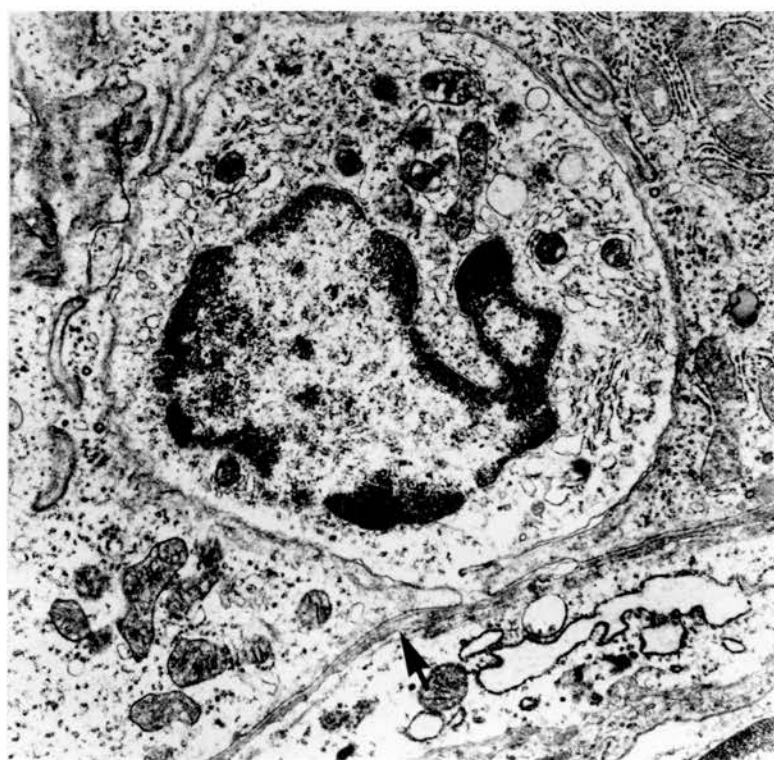
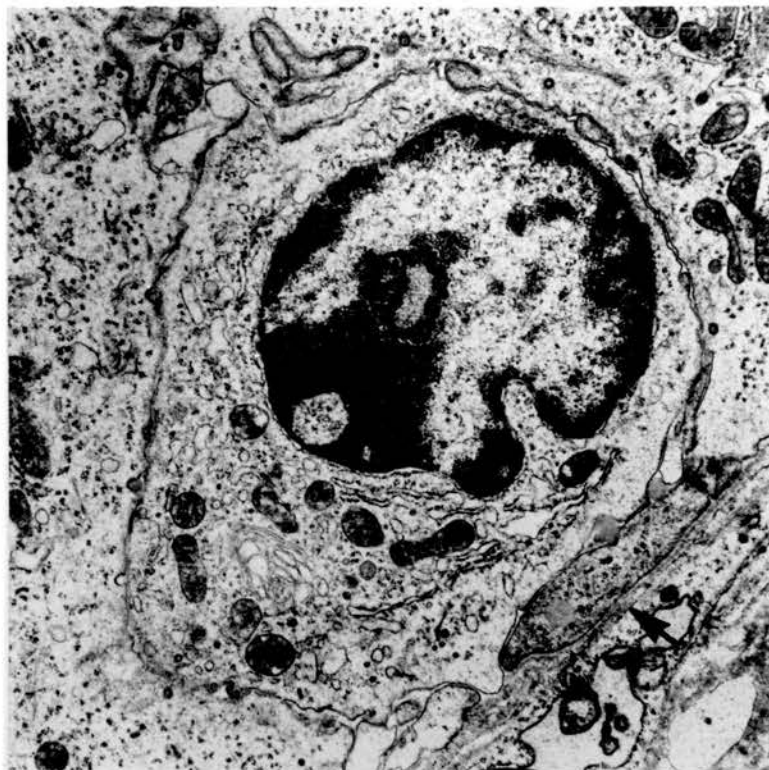


Plate 3.5 Large blast cells (BC) migrating from the lamina propria into the epithelium. This cell is characterized by a large nucleus and the presence of numerous ribosomes in the cytoplasm. The other cytoplasmic components are trailing toward the rear of the cell. Arrows indicate basement membrane.
x 7500 (approx.).

Plate 3.6 Higher magnification of the cytoplasm of a blast cell showing the clumping of ribosomes to form polysomal clusters.
x 25000 (approx.).

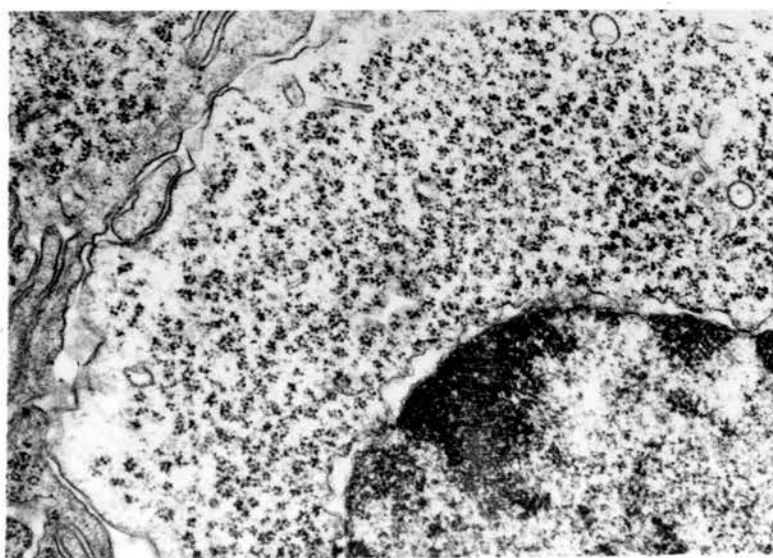
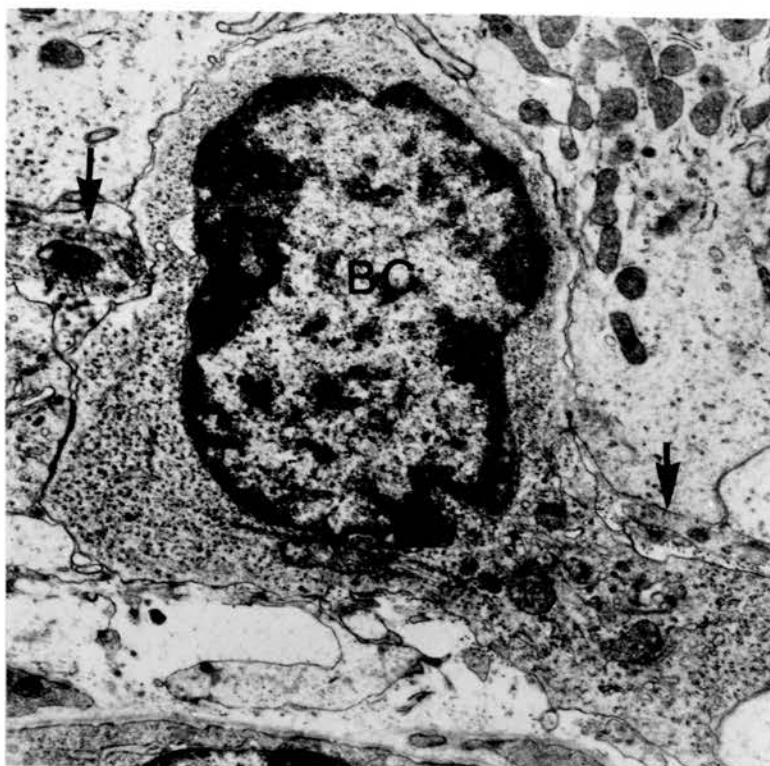


Plate 3.7 Two small lymphocytes in contact.
x 5000 (approx.).

Plate 3.8 A small lymphocyte (LC) in contact
with a blast cell (BC).
x 5000 (approx.).

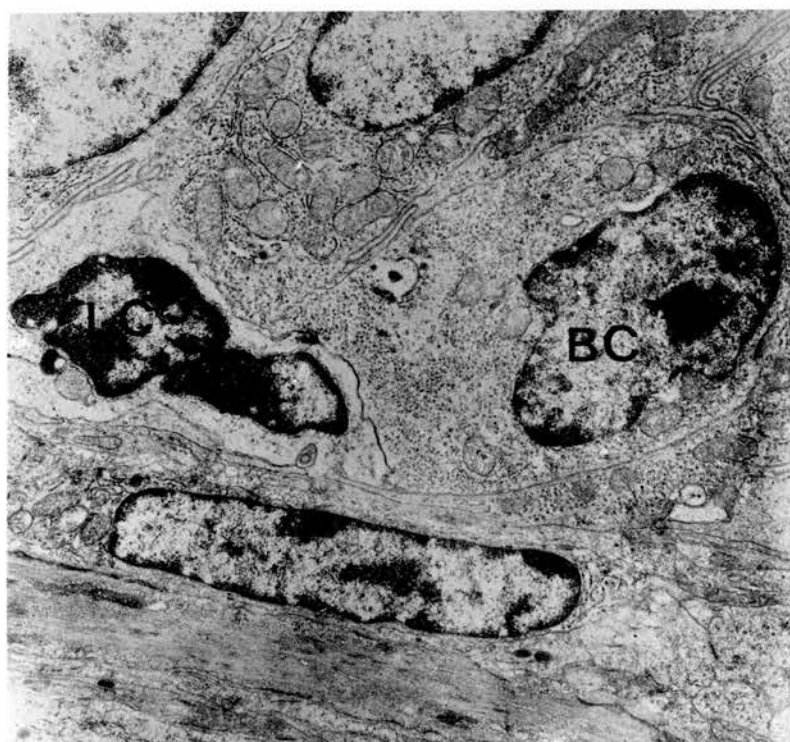
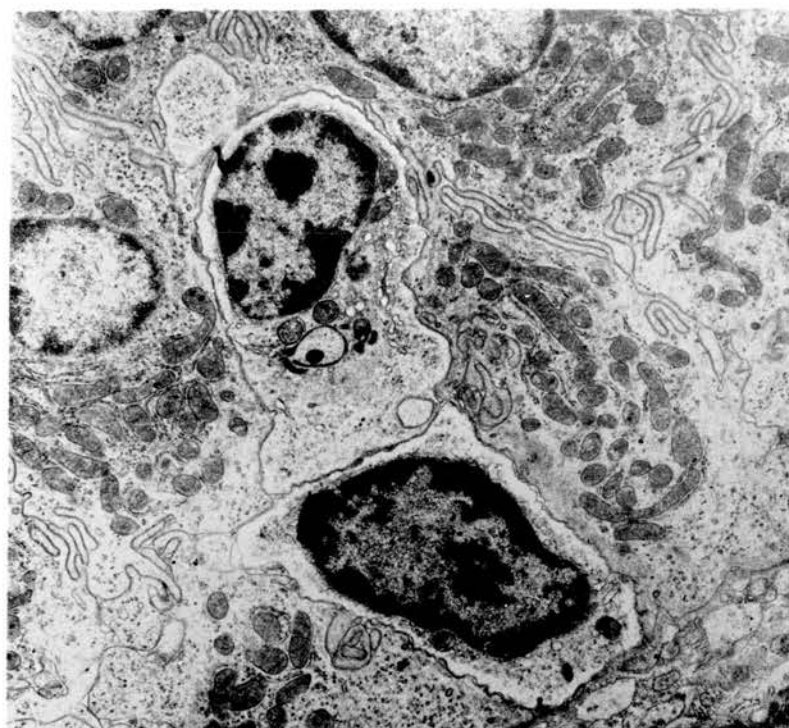


Plate 3.9 Medium sized lymphocyte with
osmiophilic cytoplasmic inclusions
at one pole of the cell.
x 7000 (approx.).

Plate 3.10 Two small-medium lymphocytes in
contact. The lower lymphocyte
contains osmiophilic cytoplasmic
inclusions at one pole. A
portion of a third lymphocyte is
present at the top centre of the
photograph.
x 8000 (approx.).

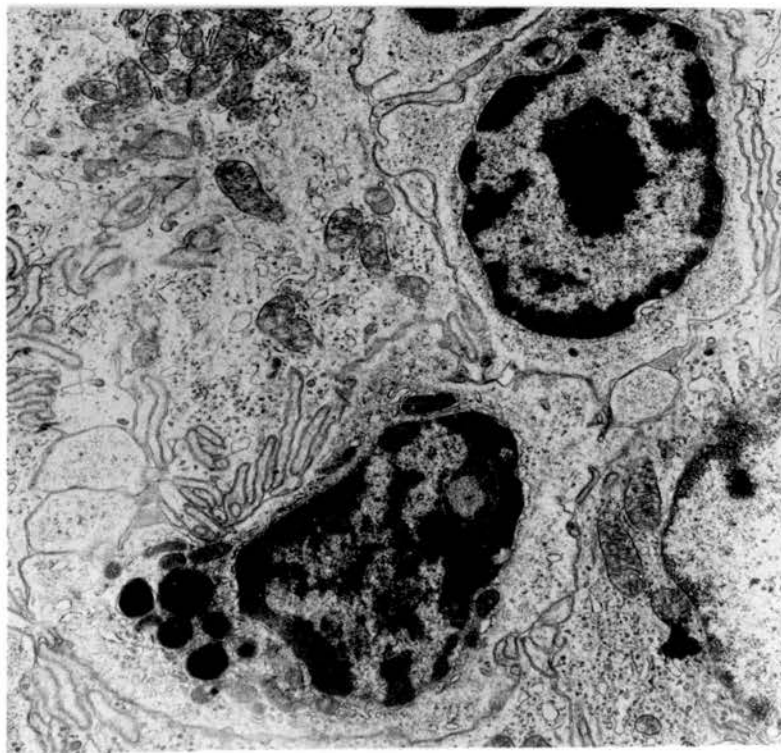
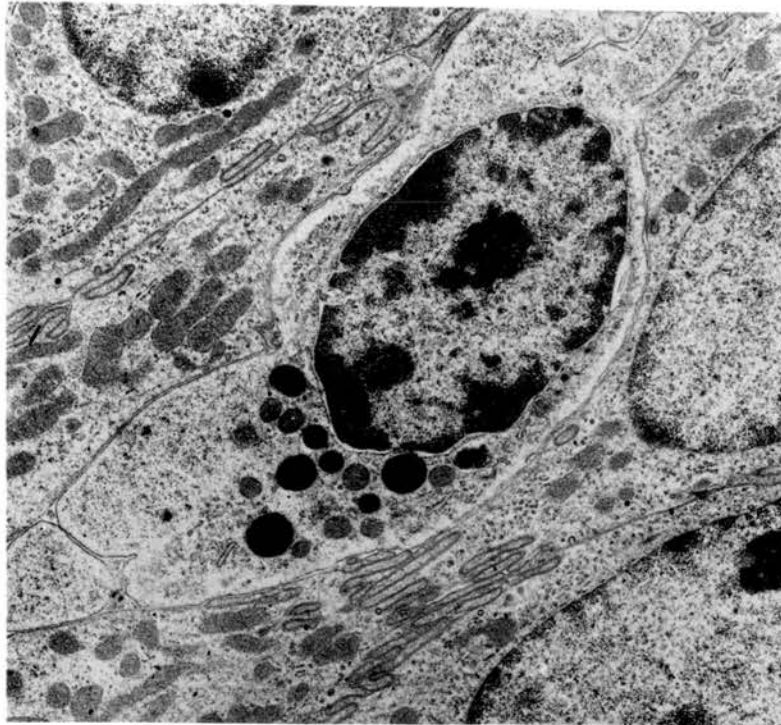
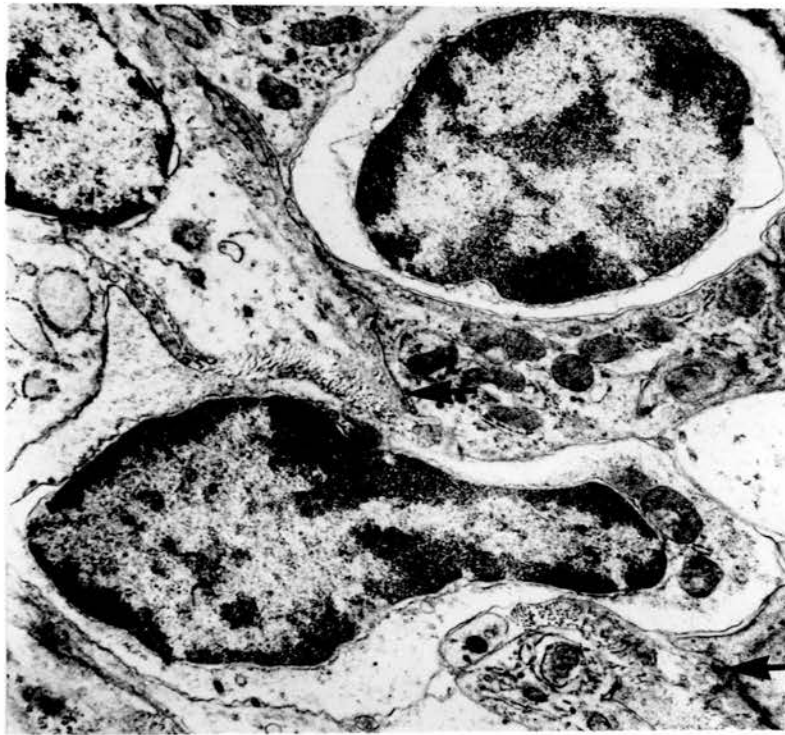


Plate 3.11 Small lymphocyte migrating from the epithelium into the lamina propria. Note the large pseudopodial extension at the anterior pole of the cell which is disrupting the basement membrane and collagen fibres in the direction of migration (arrows).
x 10000 (approx.).

Plate 3.12 A later stage in the migration of a small lymphocyte from the epithelium into the lamina propria. The lymphocyte has assumed the classical 'hand-mirror' shape with the trailing of its cytoplasmic contents towards the rear of the cell.
x 7500 (approx.).



CHAPTER 4.AUTORADIOGRAPHIC STUDIES.INTRODUCTION.

Because of the difficulties in examining lymphoid cell populations morphologically, especially those composed mainly of small lymphocytes, studies of cell kinetics in such populations have proved to be an invaluable tool for classifying and identifying the various components of body lymphocyte compartments.

One method of obtaining population profiles is by estimating the percentage of mitotic figures, a technique which has been used to compare the relative rates of proliferation in the different lymphoid organs of the rat (Andreasen and Christensen, 1949). By using colchicine, which blocks cells in metaphase, the increase, with time, in number of mitotic figures gives an estimate of flow rates. The flow rate together with the mitotic index ($\frac{\text{percentage of cells in mitosis}}{100}$) provides enough information to enable an estimate of the maximum generation time of the population to be calculated. This technique has been applied successfully to actively proliferating populations such as the bone marrow and intestinal epithelium (Dustin, 1959; Leblond, 1959). However, it can only be applied to homogeneous populations, or to heterogeneous populations in which the different compartments are easily recognized morphologically. Since most lymphoid populations are heterogeneous and the different compartments similar morphologically, the colchicine technique has been largely superseded by studies using radioactive nucleic acid precursors.

Cells incorporate phosphate during interphase and P^{32} was one of/

of the first nucleic acid precursors to be used in studies of lymphocyte kinetics (Coassin and Kline, 1957); but since then nucleic acid purine and pyrimidine precursors labelled with C^{14} or H^3 have become available and techniques based on these compounds are now the ones most commonly used. These radioactive precursors can be given as single or multiple injections and their presence in the tissue, or at least the presence of their radioactive component, can be measured by either autoradiography or scintillation counting.

The basis of the single injection technique is that the labelled precursor is available only for a short period of time after injection (less than one hour) so that cells synthesizing DNA during the availability period, or cells passing into the synthesizing phase, become labelled (Staroscik, Jenkins and Mendelsohn, 1964). It follows that the more actively proliferating populations will contain a greater number of cells in the synthesis phase, at any given time interval, than a slowly proliferating population, and therefore, a larger number of cells will be labelled with the radioactive precursor. Similarly, a rapidly dividing population will lose its label, by dilution, more rapidly than a slowly dividing one. These two characteristics make it possible to differentiate between a rapidly dividing population, with its high percentage of labelled cells and its rapid loss of label due to division, and a slowly dividing population with a low labelling index and a relatively slow rate of loss of label. It is possible to differentiate these populations in a mixture of the two types, and this makes the technique invaluable in the study of lymphocyte populations. Additions to this technique such as grain count halving and enumeration of labelled mitoses, make possible accurate/

accurate estimations of population parameters.

Studies of the kinetics of the lymphocyte population in the intestinal epithelium have been reported for the mouse (Darlington and Rogers, 1966) and for the rat (Fichtelius, 1968). In both cases the technique used was the study of labelling indices at various time intervals following a single dose of tritiated thymidine (H³T). The intervals reported by Darlington and Rogers were 1 hour, 24 hours, 36 hours, 48 hours, 5 days and 10 days, and the number of labelled cells were 0.9 per cent at 1 hour and approximately 3 per cent at all other time intervals. Using these results they calculated that the lymphocytes in the epithelium have a mean generation time between 15 and 22 days. On the other hand, Fichtelius studied the rat intestine at 30 minutes and 3 days after H³T and found that the labelling index at the tip of the villus was 2 per cent at 30 minutes and 12 per cent at 3 days. He concluded from these studies that lymphocytes synthesize DNA in situ and that they are a newly formed population. Olson (1970) found a labelling percentage in the guinea-pig intestine of 16 per cent at 1 hour and 18 per cent at 6 hours.

Clearly, there is some divergence of opinion on the kinetics of lymphocytes in the intestinal epithelium. The results of Darlington and Rogers indicate a slowly proliferating population, and those of Fichtelius indicate a population with a rapid turnover. This may be due to species differences or to sample time differences. One purpose of the present study, therefore, was to examine the labelling of the lymphocytes in the intestinal epithelium following single or multiple injections of tritiated thymidine to determine whether the cells were part of a rapidly dividing population/

population, a slowly dividing one, or whether the population was heterogeneous.

At this juncture, it is probably worthwhile to examine the population profiles reported for other lymphoid systems and to review their possible relationship to the population found in the intestinal epithelium.

The potential contribution of the thymus to the intestinal population may be adduced by the fact that thymectomy causes a depletion of this population (Fichtelius, Yunis and Good, 1968), and that a few labelled lymphocytes are found in the lamina propria and epithelium following in situ labelling of the thymus (Murray and Woods, 1964). However, following a single injection of H³T the labelling index in the thymus rises steeply to reach a peak at 3 - 4 days (Schooley, Bryant and Kelly, 1959; Metcalf, 1966; Borum, 1968), and thus, if the thymus is the source of lymphocytes in the intestinal epithelium, the peak labelling index would be expected to be found at 3 - 4 days as it is in peripheral lymphoid tissues following local labelling of the thymus (Murray and Woods, 1964; Nossal and Gorrie, 1964).

a/ The bone marrow shows an essentially similar profile to that described for the thymus, namely, a rapid increase in the labelling index to a peak at 3 - 4 days after a single injection of H³T (Osmond and Everett, 1964; Everett and Coffrey, 1966). There is also a subpopulation of transitional lymphocytes in the bone marrow whose labelling index reaches a peak at 12 - 24 hours (Osmond and Everett, 1964). Although the bone marrow seeds the peripheral lymphoid tissues (Everett and Coffrey, 1965) no data are available on the change in the numbers of labelled/

labelled cells with time, thus unlike the thymus it is not possible to predict how the labelling index of the intestinal lymphocyte population will behave if the bone marrow is its source.

If the germinal centres of lymphoid organs supply the intestinal epithelium with lymphocytes, then a very early peak in labelling index might be predicted, since following a single injection of H^3T , many of the germinal centres are 'flash' labelled and the labelling index rises steeply, reaching a peak at 6 - 12 hours (Fliedner, Kesse, Cronkite and Robertson, 1964). Although labelled cells do leave germinal centres (Yoffey, Reinhardt and Everett, 1961; Hanna, 1964), the fate of these cells is not clear, and so the question of their migration to the intestine must remain open. It is interesting to note that Miller (1964; 1965), in his studies of local labelling in the popliteal lymph node of the rat, found that labelled small lymphocytes migrated to the submucosa of the ileum, and that occasionally, small lymphocytes were seen traversing the bowel mucosa.

Although it may be possible to imply the source of cells in the intestinal epithelium by correlating the labelling profile of these cells with that of potential parent populations, other workers have adopted a more direct approach by means of cell transfer experiments.

Several experiments have been carried out with labelled thoracic duct lymphocytes. The results generally agree in that thymidine-labelled large lymphocytes seem to migrate preferentially to the lamina propria of the intestine where they resemble plasmacytic cells. A few cells also penetrate the epithelium (Porter and Cooper, 1962; Gowans and Knight, 1964; Goldschneider and McGregor, 1968; Griscelli, Vassalli and McCluskey, /



McCluskey, 1969; Hall and Smith, 1970). In contrast to newly formed lymphocytes, small long-lived uridine-labelled lymphocytes migrate to the intestine in much smaller numbers (Porter and Cooper, 1962; Gowans and Knight, 1964).

Cells from other sources, for example large thymocytes, have also been found to migrate to the intestine (Goldschneider and McGregor, 1968). Similarly, Capalbo, Makinodan and Gude (1962) found that a few thymidine-labelled spleen lymphocytes migrated to the small intestine, although the studies of Wakefield and Thorbecke (1968) would indicate that these are not derived from the germinal centres of the spleen. Occasional cells have also been found in the small intestine following injection of thymidine-labelled thymocytes (Murray and Murray, 1964). Thymidine-labelled mesenteric lymph node cells also migrate to the small intestinal mucosa (Griscelli, Vassalli and McCluskey, 1969).

In the light of these previous cell transfer experiments attempts were made to undertake cell transfer studies with mesenteric lymph node, spleen and bone marrow cell suspensions to determine whether or not these organs contributed to the lymphocyte population in the intestinal epithelium.

MATERIALS AND METHODS.

The details of the autoradiographic techniques used can be found in the Appendix (p. 251).

Experiment 1.

Eight/

Eight to nine week old C3H female mice, mean weight 24 gm, were given a single intraperitoneal injection of 25 μ c tritiated thymidine (H3T), Sp. Activity 3 - 5 curies/mMole (Radiochemical Centre, Amersham), in 0.25 ml saline. Groups of 5 - 7 mice were killed by cervical dislocation at 1, 6, 12, and 24 hours and at 2, 4, 6, 11, 18 and 25 days. The proximal 2 cm of small intestine was removed and processed for autoradiography using Ilford K2 emulsion.

Experiment 2.

C3H female mice aged 8 - 10 weeks, were given a single intraperitoneal injection of 25 μ c H3T and groups of 3 mice were killed at $\frac{1}{2}$, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, 18, 24, 36 and 48 hours after injection. The proximal part of the small intestine was removed and processed as before with Ilford K2.

Experiment 3.

C3H female mice aged 8 - 10 weeks, were given 15 μ c H3T intraperitoneally for 12 consecutive days. Groups of 3 mice were killed after 12 hours and 4, 8, 15, 25 and 38 days after the last injection and sections of the proximal small intestine processed with Ilford K2.

Experiment 4.

Ash CS1 female mice aged 8 - 10 weeks, were given 5 injections of H3T over a period of 28 hours. The dose rate used was 0.5 μ c/gm body weight. The first and last injections were given intravenously, the intervening ones intraperitoneally. One hour after the last injection the mice/

mice were killed and cell suspensions prepared from the mesenteric lymph node, spleen and bone marrow. The details of cell preparation are presented in the Appendix (p. 254). Using a ratio of 2 donors to 1 recipient, $1-3 \times 10^7$ viable cells were injected intravenously into the recipients in a volume of approximately 0.5 ml. Recipient mice were killed 3, 6, 12 and 18 hours after transfer and samples of lung, liver, kidney, spleen, mesenteric lymph node, thymus and small intestine processed for autoradiography.

Experiment 5.

C3H female mice were killed and cell suspensions of spleen and mesenteric lymph node were prepared in Medium 199 containing 10 per cent foetal calf serum. Tritiated uridine (H^3U) was added to a concentration of 5 μ c/ml, Sp. Activity 25 curies/mMole (Radiochemical Centre, Amersham), and the cells incubated for 1 hour at 37° C. After incubation the cells were spun at 1000 r.p.m. for 10 minutes, washed once with fresh 199 and resuspended for injection. Approximately $1-5 \times 10^7$ viable labelled cells were injected intravenously into Ash CS1 female mice. One recipient of spleen or mesenteric lymph node cells was killed at 45 minutes and 6, 12, 15, 20 and 24 hours. A recipient of bone marrow cells was killed at 45 minutes and 5, 12 and 20 hours. Samples of lung, liver, kidney, spleen, mesenteric lymph node, thymus and small intestine were processed for autoradiography.

RESULTS.Experiment 1.

The percentage of labelled cells was derived from counts of 200 cells per section for each of 5 non-consecutive sections per animal, except in the animals killed after 25 days where the numbers of labelled cells were so low that the sections were scanned to ensure that the result was at least a fraction of a per cent and not zero. The results presented in Figure 4.1 and Table 4.1 show that approximately 10 per cent of the cells are labelled 1 hour after injection, and that this remains constant for the next 5 hours. Between 6 and 12 hours there is a marked increase in the number of labelled cells, reaching a peak value of 23 per cent at 12 hours. After 12 hours, the numbers of labelled cells declines. This decline phase is biphasic being rapid for the first 36 hours and then much slower thereafter. Labelled cells could still be observed in the epithelium 25 days after injection.

Although no actual counts were carried out, it appeared that during the first 48 hours, heavily labelled medium to large lymphocytes were the predominant cell type labelled. A few heavily labelled large pyroninophilic cells were also encountered during the first 48 hours (Plates 4.2 and 4.3). After 4 days, only lightly labelled (usually less than 10 grains/cell) medium and small lymphocytes were seen. Labelled cells in various stages of mitosis were also seen as were labelled cells crossing the basement membrane between the lamina propria and the epithelium (Plates 4.1, 4.4 and 4.5).

TABLE 4.1.

Percentage of labelled lymphoid cells in the epithelium of the small intestinal villi of mice after a single injection of 25 μ c tritiated thymidine.

Time after H ³ T	Number of labelled cells/1000 lymphoid cells in animals number							Mean % labelled ± S.D.
	1	2	3	4	5	6	7	
1 hour	91	112	105	108	114	100	-	10.50 ± 0.85
6 hours	92	146	97	108	122	105	137	11.53 ± 2.04
12 hours	218	208	264	247	228	236	240	23.44 ± 1.86
24 hours	148	163	161	130	132	101	104	13.40 ± 2.51
2 days	73	83	52	64	63	53	-	6.47 ± 1.19
4 days	40	54	46	44	47	31	-	4.37 ± 0.77
6 days	43	27	28	25	26	-	-	2.98 ± 0.75
11 days	8	9	14	10	13	7	-	1.00 ± 0.28
18 days	9	4	11	3	3	3	-	0.55 ± 0.36
25 days	Sections scanned not counted							0.2 approx.

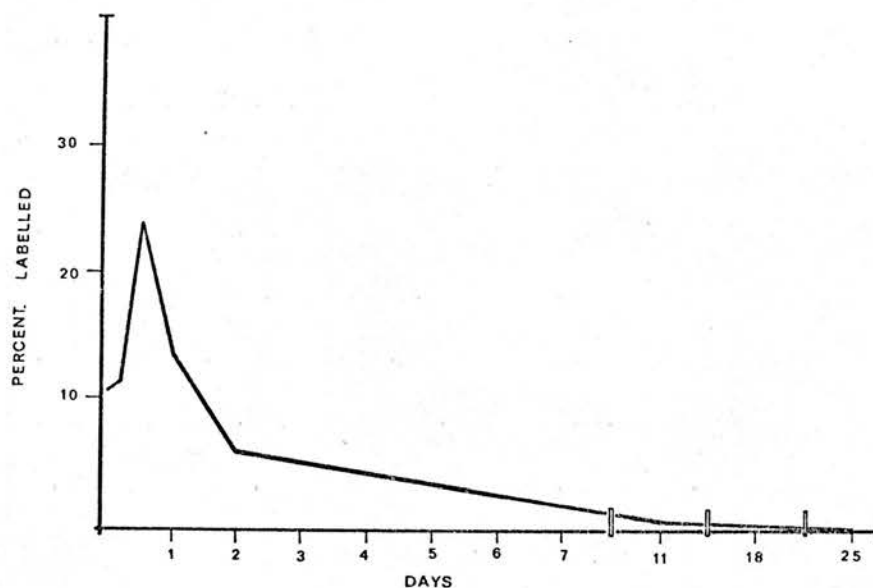


Figure 4.1 Percentage of labelled lymphoid cells in the villus epithelium at various time intervals following a single intraperitoneal injection of tritiated thymidine (1 $\mu\text{c}/\text{gm}$ B.W.).

Experiment 2.

The labelling index at close time intervals after a single injection of H3T was similar to the early phase obtained in Experiment 1 where the intervals were much greater. Approximately 10 per cent of the cells were labelled in situ and this remained so for the next 6 hours. Between 6 and 12 hours the numbers of labelled cells increased to a peak of approximately 18 per cent at 12 hours after which the numbers of labelled cells declined (Figure 4.2).

The mean grain count over the labelled cells declined rapidly over the first 10 hours after injection and then remained more or less static for the next 36 hours (Figure 4.4). The grain count halving time was in the region of 10 - 14 hours. However, the failure of the grain count to continue to fall rapidly indicates that there was probably only one significant division cycle, any subsequent division being masked by the influx of labelled cells.

Although mitotic figures were uncommon in the epithelium, sufficient numbers of villi were scanned until at least 5 mitoses had been evaluated for each animal and therefore at least 15 per time interval. From these it was possible to draw an approximate curve of the percentage of labelled mitoses (Figure 4.3). Approximately 100 per cent of mitoses are labelled 2 hours after injection and therefore $G_2 + M = 2$ hours. If the S phase is taken as the time interval between the mid-points of the ascending and descending portions of the curve then $S = 8 - 9$ hours. Since there was no distinct second peak it was not possible to estimate the total cell cycle time and thus, by subtraction, G_1 . However, if the mean grain count halving time of 10 - 14 hours is taken as the approximate cell/

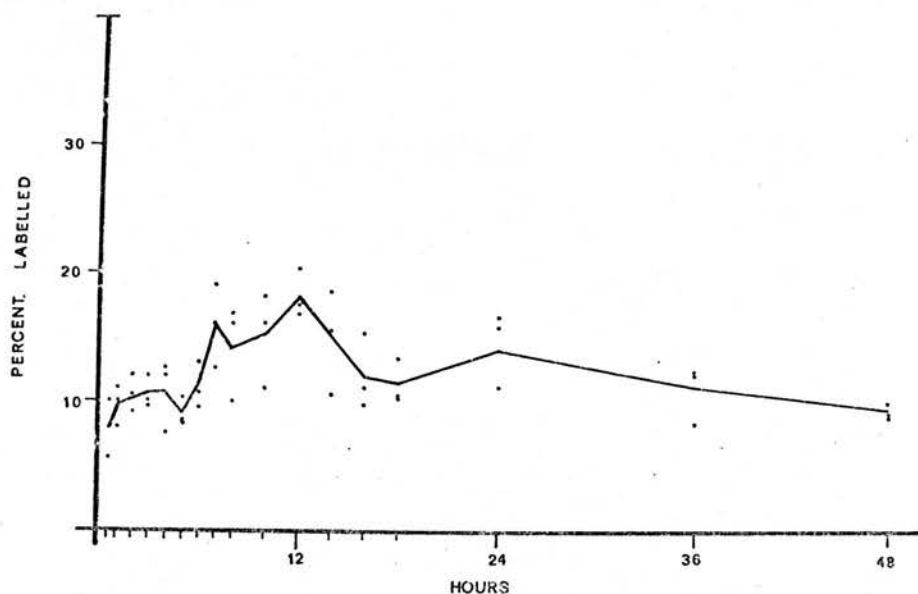


Figure 4.2 Percentage of labelled cells at close time intervals following a single injection of tritiated thymidine ($1 \mu\text{c/gm B.W. i.p.}$). Each point represents the value for a single animal, and the curve joins the mean values at each time interval.

cell cycle time then G1 is fairly short.

Thus, if the results of this short term study are considered as a whole, they indicate that a part of the population in the epithelium synthesizes DNA and then divides, and that the cell cycle time is approximately 12 - 14 hours.

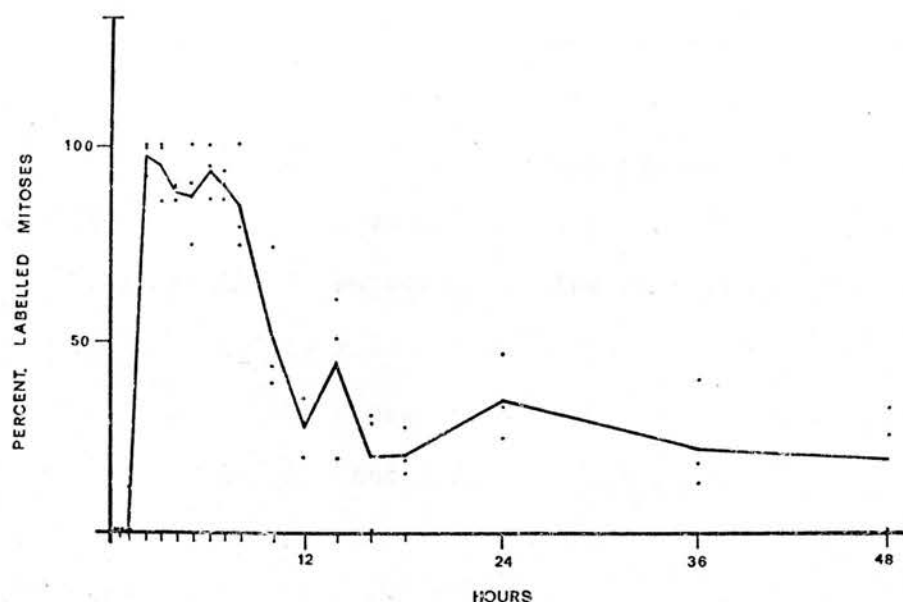


Figure 4.3 Percentage of labelled mitoses at close time intervals following a single injection of tritiated thymidine ($1 \mu\text{c}/\text{gm}$ B.W. i.p.). Each point represents the value for a single animal, and the curve joins the mean values at each time interval.

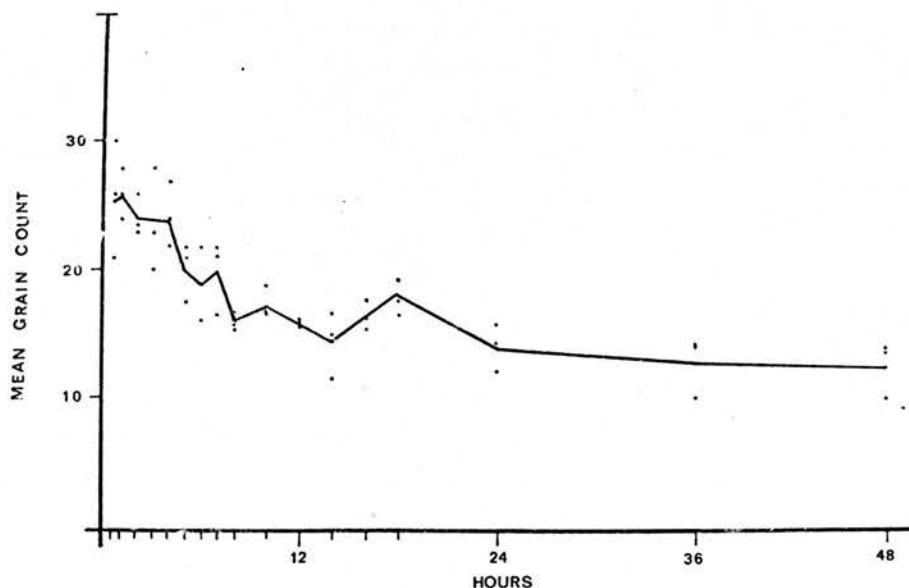


Figure 4.4 Mean grain count at close time intervals following a single injection of tritiated thymidine ($1 \mu\text{c}/\text{gm}$ B.W. i.p.). Each point represents the value for a single animal, and the curve joins the mean values at each time interval.

TABLE 4.2.

Percentage labelled cells, and mean grain counts over these cells at close time intervals after a single intraperitoneal injection of H³T. The number of labelled mitoses/number examined is also presented.

Time after injection (hr.)	% labelled cells in mouse number			Mean grain count in mouse number [±]			Labelled mitoses in mouse number		
	1	2	3	1	2	3	1	2	3
$\frac{1}{2}$	5.5	8.0	10.0	30.0	21.0	26.0	0/5	0/9	0/8
1	10.0	11.0	8.0	24.0	26.0	28.0	1/8	0/5	0/5
2	10.5	12.0	9.0	23.0	23.5	26.0	8/8	13/14	5/5
3	10.0	12.0	9.5	23.0	28.0	20.0	8/8	5/5	6/7
4	7.5	12.0	12.5	22.0	24.0	27.0	6/7	7/8	7/8
5	8.5	8.5	10.0	21.0	22.0	17.5	6/8	4/5	5/5
6	10.5	9.5	13.0	19.0	22.0	16.0	6/7	8/8	19/20
7	19.0	12.5	16.0	22.0	21.5	16.5	12/14	12/13	4/5
8	10.0	16.0	16.5	16.5	15.5	15.0	8/8	4/6	12/15
10	11.0	18.0	16.0	16.5	18.5	16.5	4/6	6/16	4/9
12	17.5	16.5	20.5	16.0	15.5	16.0	5/18	1/5	3/8
14	18.5	10.5	15.5	16.5	11.5	15.0	5/8	1/5	3/6
16	9.5	15.0	11.0	17.5	15.5	16.0	0/7	3/9	3/10
18	10.5	10.5	13.5	17.5	16.5	19.0	3/16	2/13	3/11
24	11.5	16.5	15.5	14.0	12.0	15.5	3/9	6/13	3/12
36	8.5	12.0	12.5	10.0	14.0	14.0	1/8	2/5	1/6
48	9.0	10.0	8.5	10.0	14.0	13.5	0/9	3/9	1/5

[±] Figures derived from examination of 200 cells in the epithelium/animal.

Experiment 3.

The percentage of labelled cells and mean grain count following injection of H3T for 12 consecutive days are shown in Table 4.3 and Figures 4.5 and 4.6. The change in the number of labelled cells appeared to show 3 phases of decline. There was a rapid decline in the first 4 days, a slower one over the next 3 weeks and an even more gradual decline over the final 2 weeks of the experiment. The mean grain count decreased slightly over the first 4 days and remained more or less static thereafter.

TABLE 4.3.

Percentage of labelled cells and mean grain count after intraperitoneal injection of H3T for 12 consecutive days.

Time after injection (days)	% labelled cells in mouse number			Mean grain count in mouse number		
	1	2	3	1	2	3
0.5	46.5	41.0	50.5	13.9	14.0	14.3
4	23.0	24.0	29.0	11.1	12.1	11.4
8	19.0	17.0	22.0	12.0	10.7	13.7
15	12.0	14.0	12.0	10.7	11.4	11.5
25	3.0	2.0	3.0	10.0	12.7	10.5
38	1.5	1.25	1.25	10.6	9.5	10.1

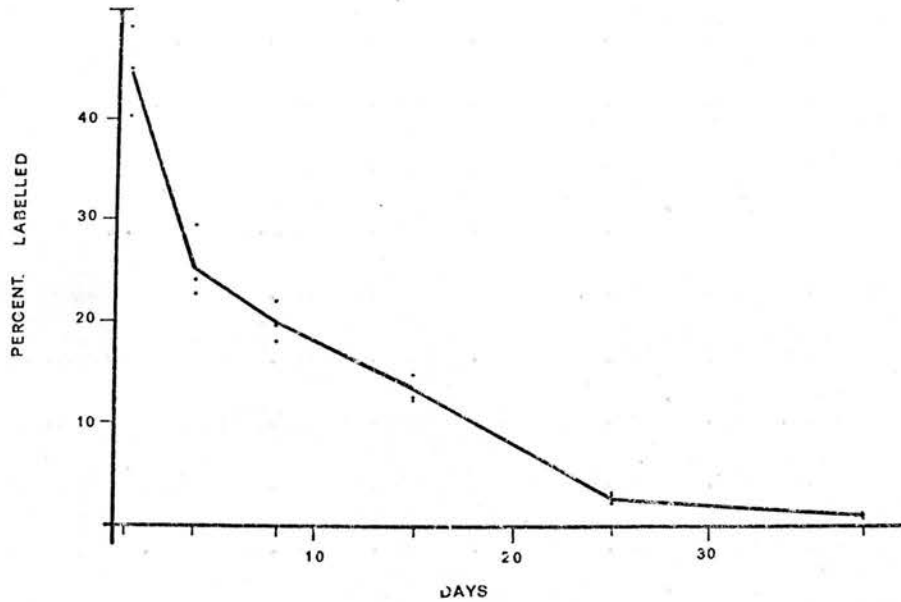


Figure 4.5 Percentage of labelled cells after injection of tritiated thymidine for 12 consecutive days. Each point represents the value for a single animal and the curve joins the mean values at each time interval.

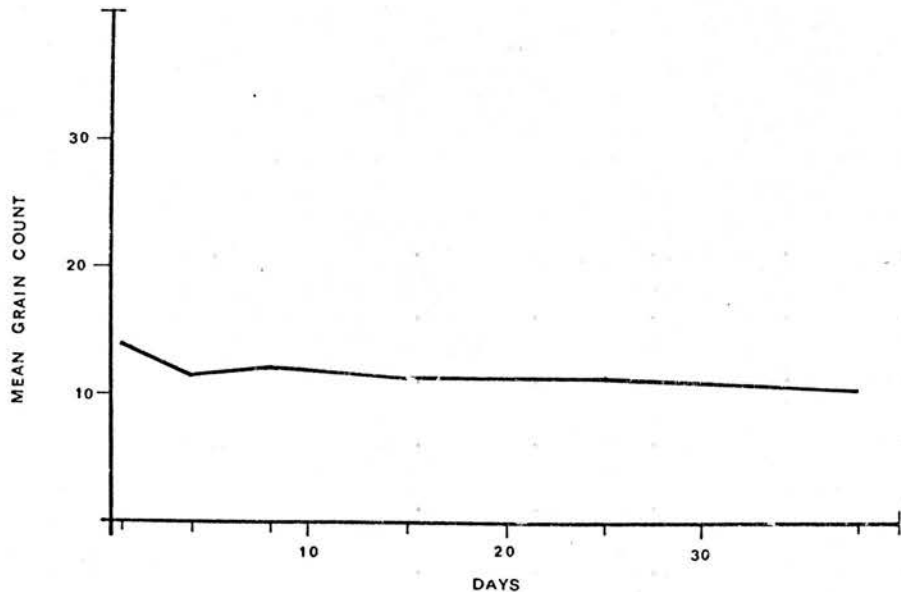


Figure 4.6 Mean grain count over labelled cells after multiple injection of tritiated thymidine for 12 consecutive days. The curve joins the mean value for 3 animals at each time interval.

Experiment 4.

Multiple injections of H³T into donor mice labelled between 10 - 30 per cent of the lymphoid cells. Fewer labelled cells were found in smears of mesenteric lymph node cells than bone marrow. The spleen occupied an intermediate position.

Three hours after transfusion, labelled cells were found mainly in the lung and the liver of the recipient with occasional cells being found in the spleen and mesenteric lymph node. By 6 hours, the number of labelled cells in the spleen and mesenteric lymph node had increased considerably, especially so in the spleen. At 12 and 18 hours, sections of spleen were found to contain the greatest number of labelled cells.

Labelled cells were found only occasionally in sections of small intestine (usually less than 1 cell/10 villus units). They were found in both the lamina propria and the epithelium of sections taken from all time intervals, regardless of the source of transfused cells, but were more frequent at 12 and 18 hours after transfusion and more numerous in animals receiving lymph node than those receiving spleen and bone marrow. The cells seen were generally large pyroninophilic cells in the lamina propria, although some non-pyroninophils were found in the epithelium.

Experiment 5.

Incubation of bone marrow, spleen or mesenteric lymph node lymphoid cells with H³U in vitro resulted in the labelling of usually more than 90 per cent of the cells.

Within 1 hour after injection, labelled cells were found in large/

large numbers in the lung, liver, spleen and mesenteric lymph node. Between 1 - 24 hours the numbers in the lung and liver declined, and at 24 hours large numbers of labelled cells were present only in the mesenteric lymph node and spleen.

By comparison with the number of labelled cells found in the spleen and lymph node, cells were found only occasionally in the small intestine. Cells were found with equal frequency in the intestine when bone marrow and mesenteric lymph node cells were injected (3 - 10/10 villus units), whereas fewer cells were found following spleen cell injection (usually below 1/10 villus units). Cells were most commonly found in the lamina propria, but a few cells were also seen in the epithelium, and at least a few cells were found at all the time intervals studied. Both large and small labelled cells were found in the intestine.

DISCUSSION.

Following a single injection of H³T, a study of the labelling index over the next 25 days has shown that approximately 10 per cent of the cells are synthesizing DNA in situ. The number of labelled cells remains more or less constant for the next 6 hours and then increases rapidly, reaching a peak at 12 hours. From 12 hours onwards, the number of labelled cells decreases. The decline phase is biphasic, falling rapidly between 12 and 48 hours and slowly thereafter. Labelled lymphoid cells are still present 25 days after H³T injection. On the basis of the labelling index profile, and particularly the biphasic decline curve, it/

it was concluded from Experiment 1 that the lymphoid cell population in the epithelium is composed of short-lived and long-lived subpopulations. Similar biphasic decline curves representing short-lived and long-lived components of lymphoid cell populations have been described for blood (Fichtelius, 1953; Ottesen, 1954; Hamilton, 1956; Coassin and Kline, 1957; Resegotti, 1957), thoracic duct lymph (Caffrey, Rieke and Everett, 1962; Rieke and Schwarz, 1966) and lymph node (Rieke, Caffrey and Everett, 1963; Craddock, Nakai, Fukuta and Vanslager, 1964; Miller, 1964).

Since Experiment 1 had shown that the population was heterogeneous, Experiments 2 and 3 were carried out to investigate the various components more closely. Experiment 2 confirmed part of the results obtained in Experiment 1, namely, that approximately 10 per cent of the cells synthesize DNA in situ, and that no significant increase in the number of cells occurs until 6 hours after injection of H3T. The increase between 6 - 12 hours can be attributed to 2 factors, division of labelled cells in the epithelium, evidenced by the presence of labelled mitoses, and migration of labelled cells into the epithelium from the lamina propria, as indicated in Plates 4.4 and 4.5. Thus, newly formed cells in the epithelium occur either as a result of cell division in situ or by migration of newly formed cells into the epithelium from an external source. From a study of the change in the percentage of labelled mitoses with time, and to a lesser extent from the change in mean grain count, it is concluded that the population of cells synthesizing DNA and dividing in situ have a total cell cycle time of 10 - 14 hours.

Examination of the labelling percentage and mean grain count following multiple injections of H3T revealed two main facts. First of all, /

all, the decline phase appears to have 3 components, a rapid phase over the first 4 days, a slower phase over the next 3 weeks and a very slow decline over the last 2 weeks. Whether this decline truly indicates 3 different cell populations, or is just an artefact representing graphically the accumulation of several decline phases produced by spreading the H3T injections over 12 days is unknown. However, it does confirm the fact that part of the lymphocyte population is long-lived, that part is short-lived, and that there is possibly a medium-lived population as well. The second observation, namely the very slow decline in mean grain counts indicates that the longevity of the remaining labelled lymphocyte population is in the order of months rather than days.

The present results may explain the different conclusions reached by Fichtelius (1968) and Darlington and Rogers (1966). Fichtelius concluded that the lymphocytes in the intestinal epithelium of the rat were a newly formed population, whereas Darlington and Rogers found that in the mouse they were slowly proliferating. Fichtelius' conclusion was derived from a short term study over 3 days after H3T injection, whereas Darlington and Rogers based their conclusion on data obtained up to 10 days after labelling, but omitting the critical period between 1 and 24 hours. Thus, apparent species difference in lymphocyte kinetics may be attributable to differences in sampling time, but further work will be necessary to confirm this point.

The cell labelling profile obtained in these experiments is similar to that found in two other lymphoid cell systems, germinal centres and delayed hypersensitivity reactions. Single pulse labelling of germinal centres produces a peak labelling index at 6 - 12 hours and from 24/

24 hours onwards there is rapid decline until by 6 days only a few labelled cells are found (Fliedner, Kesse, Cronkite and Robertson, 1964). These workers also suggested a generation time of 13.4 hours, a result similar to that found in this study. However, similar generation times have also been found for plaque-forming cells (Tannenberg and Malaviya, 1968), for cells in antigen stimulated spleens (Capalbo and Makinodan, 1964) and for plasma cell precursors (Schooley, 1961; Nossal and Makela, 1962), so that it is not specifically a feature of germinal centre cells.

Following a single injection of H^3T , 5 - 10 per cent of cells participating in delayed hypersensitivity reactions are labelled at 1 hour and the labelling index then rises steeply to a peak at 12 - 24 hours, remaining relatively high for another 24 hours (Kosunen, Waksman, Flax and Tihen, 1963; Kosunen, Waksman and Samuelson, 1963; Groth, 1967). The plateau phase between 24 - 48 hours found in delayed hypersensitivity reactions in other sites is probably due to retention of labelled cells in the lesion, whereas the decline found in the intestinal epithelium could be explained by assuming that labelled cells are more likely to be lost from the lesion into the lumen of the intestine.

The cell transfer experiments met with only limited success. When thymidine or uridine-labelled cells were injected, relatively few cells were found in the intestine compared with the numbers found in the organized lymphoid organs such as the spleen, lymph node and Peyer's patches. It was noticeable, however, that cells were usually more readily found when mesenteric lymph node cells were transfused rather than spleen or bone marrow cells. However, under the conditions of these experiments, the only conclusion that can be drawn is that there is no large/

large scale migration of cells to the intestine from any of the sources injected, but that all the sources appear to contribute a small number of cells. These results would therefore agree with those of other workers who found that a few cells migrated to the intestine when thymocytes (Murray and Murray, 1964), spleen cells (Capalbo, Makinodan and Gude, 1962) and long-lived thoracic duct small lymphocytes (Porter and Cooper, 1962; Gowans and Knight, 1964) are injected. Thus, there may be no single source of lymphocytes in the intestine, the heterogeneity found in both the morphology and cell kinetic profile being a reflection of the multiple origins of these cells. Since there is a considerable interchange between different body lymphoid cell compartments (Yoffey, 1967), the findings that several sources may contribute to the intestinal lymphocyte population may not be entirely unexpected.

There are two possible alternatives, however, to the multiple source hypothesis. The first of these is that a source of cells not included in this study is the primary source of intestinal lymphocytes. The second is that one of the sources studied is, in fact, the primary source, but that handling the tissues in vitro interferes with the migration pattern of the cells.

One alternative source of cells is the thymus, but since only a few cells migrate to the intestine when thymus cells are injected (Murray and Murray, 1964) this organ can be excluded. A more likely alternative is the intestine itself and its associated lymphoid tissues. It has been well established that large DNA synthesizing cells in the thoracic duct lymph migrate to the lamina propria of the intestine (Gowans and Knight, 1964; Goldschneider and McGregor, 1968; Griscelli, Vassalli and/

and McCluskey, 1969). Since a large proportion of thoracic duct lymph is derived from the intestine (Mann and Higgins, 1950), the intestine or its associated lymphoid tissues are probably a major source of these cells. It was noted in this study that the greatest numbers of thymidine-labelled cells were found following injection of labelled mesenteric lymph node cells. Griscelli, Vassalli and McCluskey (1969) also found that thymidine-labelled mesenteric lymph node cells migrate to the intestine. Although the Peyer's patches also contribute cells to the thoracic duct lymph, no studies have been undertaken to examine their distribution after injection of cell suspensions and thus no conclusion can be drawn about the contribution of Peyer's patches to the lymphocyte population in the intestinal mucosa. Finally, the lymphocytes in the epithelium itself synthesize DNA and divide and they may also contribute cells to the thoracic duct lymph via the lacteals.

Thus, on this evidence it is possible to postulate a circulation system in which newly formed or DNA synthesizing cells leave the intestine or its associated lymphoid tissues, enter the thoracic duct lymph, and then migrate via the blood stream back to the intestine. This has led to the hypothesis that the migration of large lymphocytes to the intestine via the thoracic duct lymph is part of a local defence response to intestinal antigens resulting in the local production of antibody, notably IgA (Gowans and Knight, 1964; Griscelli, Vassalli and McCluskey, 1969). However, although this hypothesis may be true, some doubt is cast upon it by the findings that large cells from the thymus (Goldschneider and McGregor, 1968), and large cells in the thoracic duct lymph derived mainly from a non-intestinal source (Hall and Smith, 1970) also show a preferential/

preferential migration to the small intestine. Therefore the preferential migration of large lymphocytes to the intestine following intravenous injection may be a non-specific phenomenon and this raises the whole question regarding the validity of cell transfer techniques as a method of outlining in vivo migration streams.

The second alternative to the multiple source hypothesis, namely that the primary source of cells is susceptible to environmental changes, is really a continuation of the argument that cell transfer techniques may produce artificial results when compared with actual in vivo techniques. The labelling index profile found in Experiments 1 and 3 indicates that there is a long-lived lymphocyte population in the epithelium. This conclusion is supported by the fact that thymectomy causes a significant decrease in the numbers of these cells (Fichtelius, Yunis and Good, 1968), and that there is a fairly substantial corticosteroid resistant population (see Chapter 7). However, when uridine-labelled thoracic duct small lymphocytes are injected, very few migrate to the intestine, the majority accumulating in lymphoid tissues (Porter and Cooper, 1962; Gowans and Knight, 1964). The studies with uridine-labelled cell suspensions reported here agree with those observations in that very few cells were found in the intestine when compared with the numbers of cells accumulating in the organized lymphoid tissues. Thus, there appears to be a lack of correlation between in vivo analysis and cell transfer studies.

The possibility that in vitro handling may interfere with subsequent cell migration is suggested by the results reported by various workers (Bradfield and Born, 1969; Gesner, Woodruff and McCluskey, 1969; Woodruff/

Woodruff and Gesner, 1969). These workers found that compounds such as heparin, trypsin or neuraminidase which have cell surface effects interfere with the circulation of small lymphocytes. Gesner, Woodruff and McCluskey (1969) also found that migration processes to different tissues may depend on different mechanisms since trypsin will prevent homing to a lymph node, and at the same time, migration to the spleen white pulp is normal. It may be therefore that the migration process to the intestine is particularly sensitive to disruption by in vitro manipulation.

Host factors as well as in vitro manipulation may also modify the circulation of lymphocytes. Thus, the circulation of lymphocytes is modified in mice injected with Bordetella pertussis (Morse and Barron, 1970), and in animals treated with ACTH, corticosteroids, pyrogens, histamine and antigens (Schnappauf and Schnappauf, 1968). Interference with lymphocyte circulation in Experiments 4 and 5 may therefore be suspected since the use of non-syngeneic animals, and the presence of foreign proteins in the medium respectively, may produce cell surface changes in response to antigenic stimulation. Stress responses in the recipient following the injection of a relatively large volume of cells may also interfere with the distribution of the injected cells. Therefore, the failure of transfused spleen, bone marrow or lymph node cells to migrate to the intestine in large numbers does not rule out the possibility that one of these organs is the primary source of lymphocytes in the epithelium in vivo.

To summarise, a study of the numbers of labelled cells in the epithelium following administration of H³T indicates that the population is/

is composed of both long-lived and short-lived cells and includes a dividing cell population. The labelling index profile obtained was similar in some respects to that reported for germinal centre cells and for delayed hypersensitivity reactions. Cell transfer experiments were unable to establish any major source of cells contributing to the intestinal epithelial lymphocyte population, but indicated that a few cells migrated to the intestine regardless of the source injected, and that the mesenteric lymph node contributed more newly formed cells than either the spleen or bone marrow.

Plate 4.1 Labelled lymphocytes in various
 stages of mitosis.
 Methyl Green-Pyronin x 1000 (approx.).

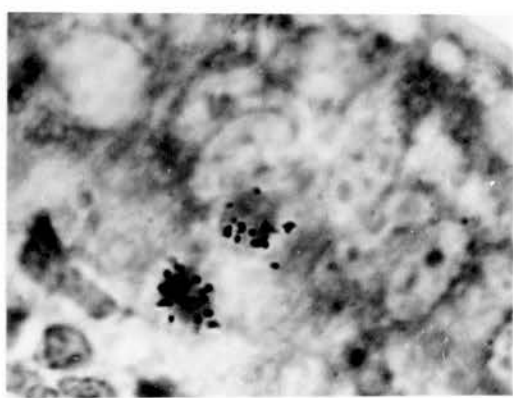
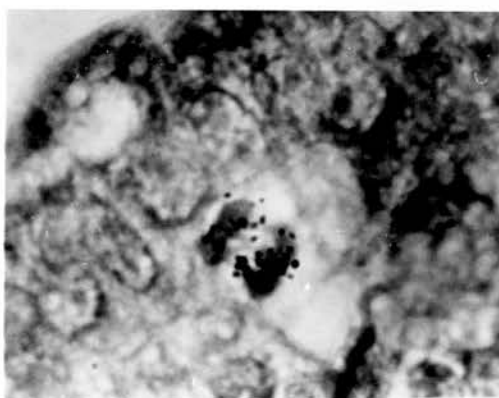
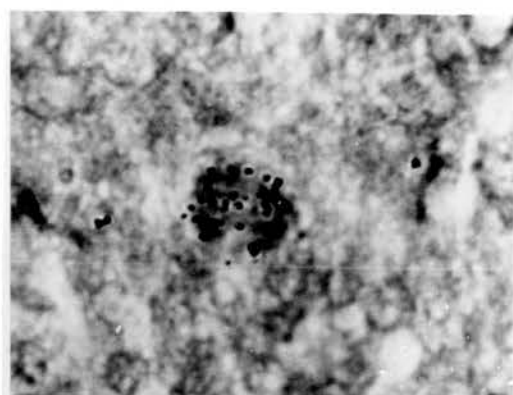
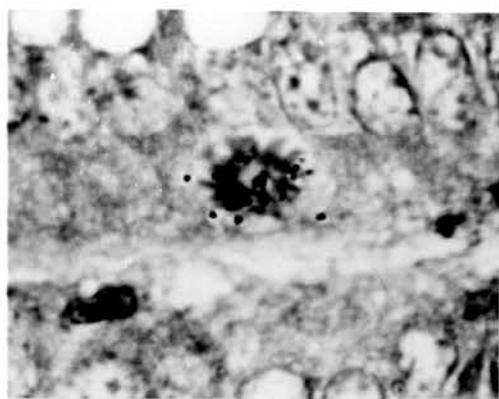


Plate 4.2 Labelled large lymphocyte 12 hours
 after tritiated thymidine
 (1 μ c/gm B.W. i.p.).
 Methyl Green-Pyronin x 1400 (approx.).

Plate 4.3 Labelled large pyroninophilic
 cell in the villus epithelium
 12 hours after tritiated thymidine.
 An unlabelled pyroninophilic cell
 is also present.
 Methyl Green-Pyronin x 1400 (approx.).

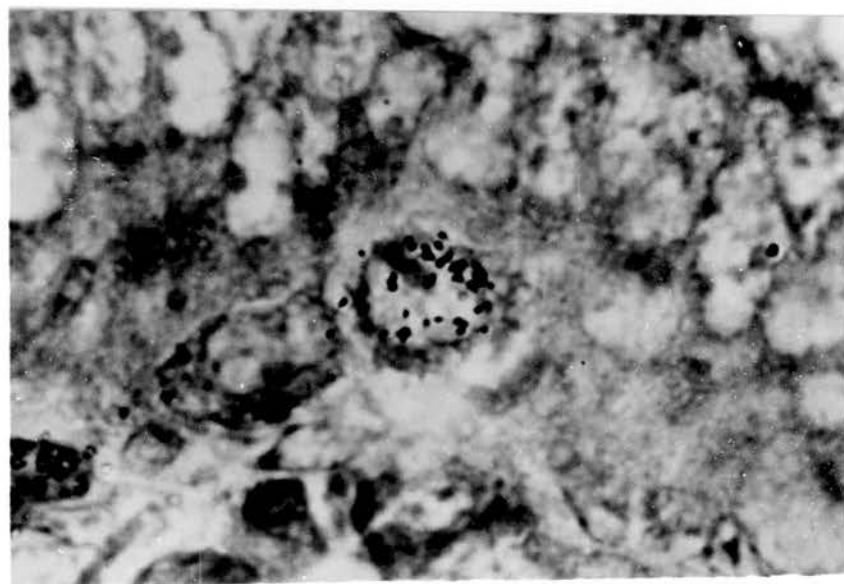
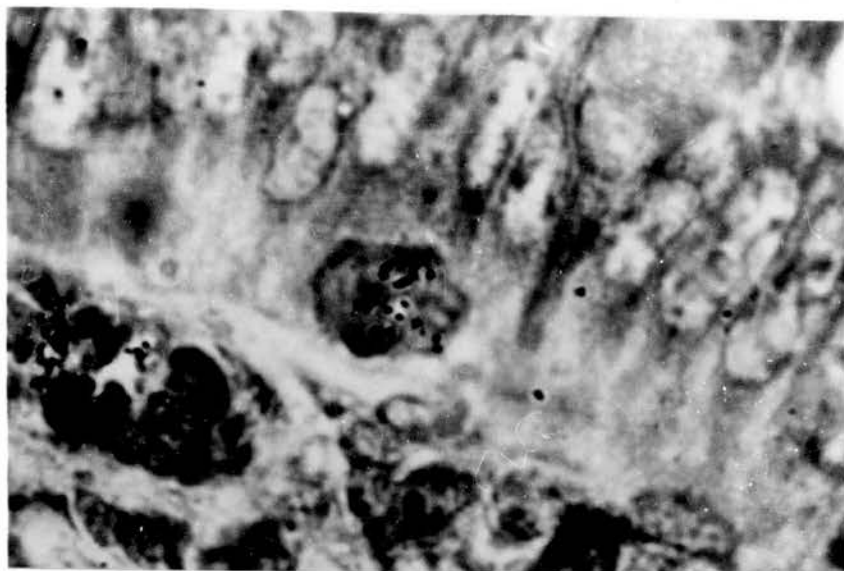
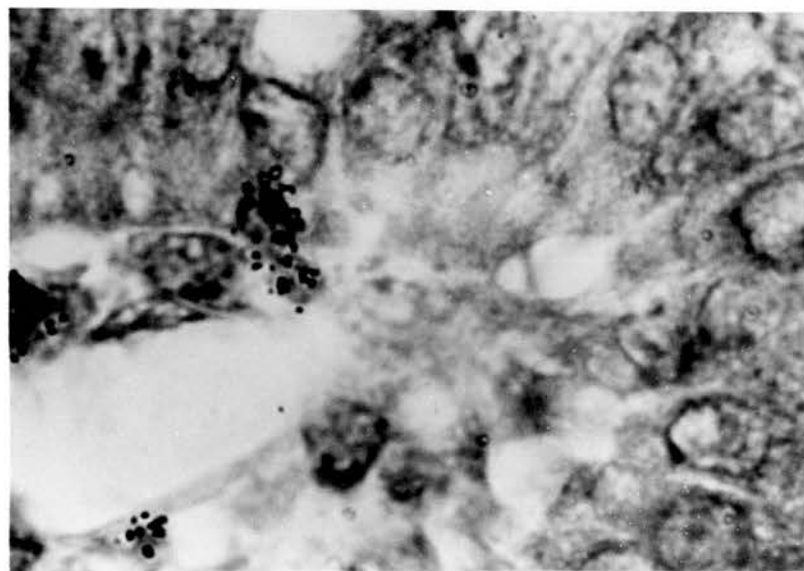
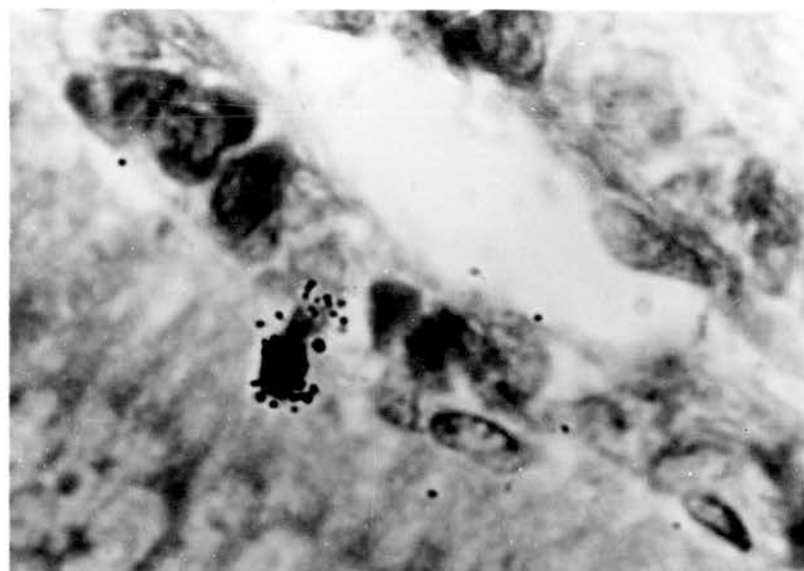


Plate 4.4 Labelled small-medium lymphocytes
 and crossing the basement membrane
Plate 4.5 between the epithelium and lamina
 propria 12 hours after a single
 injection of tritiated thymidine.
 Methyl Green-Pyronin x 1400 (approx.).



CHAPTER 5.A. EFFECT OF AGE.INTRODUCTION.

On the basis of autoradiographic studies on lymphocytes in the villus epithelium of the rat intestine, and on studies of the phylogeny of these cells in vertebrates, it has been suggested that the association between lymphocytes and villus epithelial cells may represent a diffuse equivalent of the avian bursa of Fabricius (Fichtelius, 1967; Fichtelius, 1968; Fichtelius, Finstad and Good, 1968; Fichtelius, Finstad and Good, 1969). It is important, therefore, to determine whether or not the ontogeny of this 'diffuse lympho-epithelial tissue' also supports the hypothesis that it functions as a primary or central lymphoid organ.

The ontogeny of the known primary lymphoid organs, the thymus in the mammal and the thymus and bursa of Fabricius in the fowl, has characteristic features which serve to differentiate these organs from the peripheral lymphoid tissues. These organs arise from endodermal-mesenchymal interaction in the early embryo and are well developed at birth (Ackerman and Knouff, 1959; Papermaster and Good, 1962; Ball, 1963; Ortega, Kattine and Spurlock, 1965; Hess, Stoner and Cottier, 1967; Chen and Ruth, 1969; Kruml, Ludvik, Trebichavsky, Mandel and Kovaru, 1969). The maximum growth rate of these organs is in the first 2 - 3 weeks after birth, with subsequent atrophy as the animals mature, the atrophy of the bursa being more complete than that of the thymus (Riddle, 1928; Glick, 1956; Glick, Chang and Jaap, 1956; Glick, 1960; Axelrad and van der Gaag, 1962; Ball, 1963; Metcalf, 1964; Borum, 1965; Hess, Stoner and Cottier, /

Cottier, 1967; Clark, 1968). Finally, the development of the thymus and bursa is independent of antigenic stimulation, and is similar in conventional and germ-free animals (Thorbecke, Gordon, Wostman, Wagner and Reyniers, 1957; Kruml, Ludvik, Trebichavsky, Mandel and Kovaru, 1969).

The object of this study was to determine whether or not the lymphocytes in the villus epithelium of the mouse intestine have a similar ontogeny to those described in other primary lymphoid organs, namely, are well developed at birth, show maximum development during the first 3 weeks of life and then commence to atrophy as sexual maturity is approached. The question of an ontogeny independent of antigenic stimulation is considered in Section B.

MATERIALS AND METHODS.

Three male and 3 female mice were killed for histology at 1 - 2 days, and 1, 2, 4, 6 and 10 weeks of age. The number of lymphocytes per 1000 epithelial cells was determined in 5 sections from a segment of proximal small intestine from each mouse.

RESULTS.

The lymphocyte population in the villus epithelium was poorly developed for the first 2 weeks after birth. A significant rise in the number of lymphocytes per 1000 epithelial cells occurred between 2 and 4 weeks, and their numbers continued to rise until at least 10 weeks of age (Figure 5.1; Table 5.1).

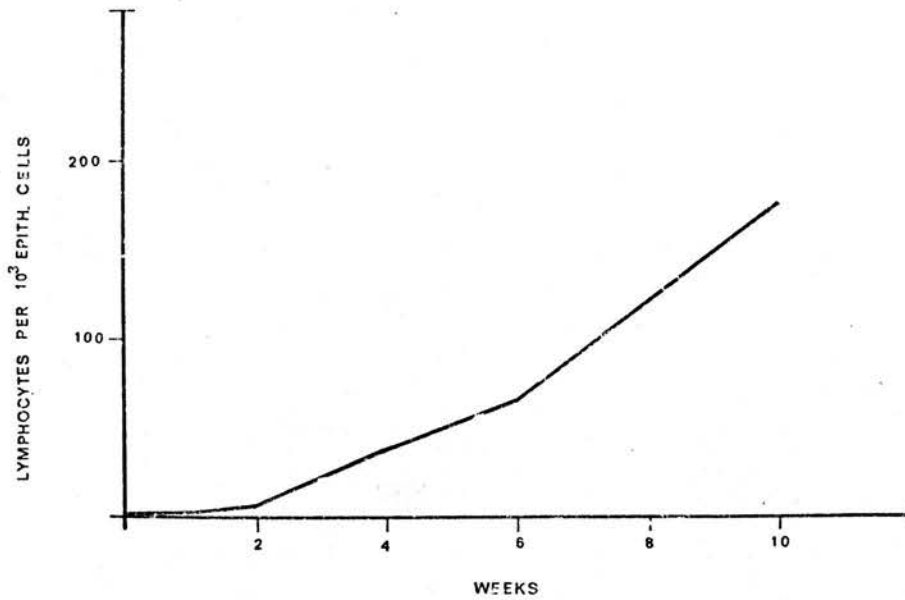


Figure 5.1 The number of lymphocytes in the intestinal epithelium of mice between birth and 10 weeks of age.

TABLE 5.1.

Number of lymphocytes per 1000 epithelial cells in sections of villi of conventional mice at different ages.

Age in weeks	Number of lymphocytes per 1000 epithelial cells in mouse number *						Mean \pm S.D.
	1	2	3	4	5	6	
Newborn	0.4	1	0.8	0.4	1.2	0.4	0.7 ± 0.3
1 week	2.4	1.0	2.0	1.8	1.8	2.8	2.0 ± 0.6
2 weeks	7	6	7	6	6	8	6.7 ± 0.8
4 weeks	34	35	38	35	39	35	36.0 ± 2.0
6 weeks	62	63	54	78	63	77	66.2 ± 10.0
10 weeks	196	199	207	114	190	151	176.2 ± 36.0

* Mean of 5 counts in non-consecutive sections.

DISCUSSION.

The results show that the lymphocyte population in the intestinal epithelium is poorly developed at birth, and does not increase significantly until after 2 weeks of age. These results are consistent with those reported by Fichtelius, Yunis and Good (1968) and do not support the hypothesis that the diffuse lymphocyte population in the villus epithelium is the mammalian equivalent of the avian bursa of Fabricius. With the exception that they represent a lymphocyte-epithelial cell association, the criteria outlined by Good, Gabrielsen, Peterson, Finstad and Cooper (1966) are not fulfilled:- 'The primordial tissue for the mammalian immunoglobulin producing tissue is presumed to be another gut-associated, glandular-lymphoid tissue, lympho-epithelial in type, relatively large in the embryo and neonate and involuting about the time of sexual maturity'.

Although the results are not consistent with the hypothesis of a bursal equivalent, the relatively late neonatal development of the intestinal epithelial lymphocyte population is associated with the development of the peripheral lymphoid tissue, and with other physiological changes which take place in young mice (and rats) between 2 and 4 weeks of age.

Thus, the peripheral lymph nodes are poorly developed at birth, but morphologically mature lymph nodes appear at 2 weeks of age and by 4 weeks of age, full structural maturity of mouse lymph nodes is usually seen (Argyris, 1963; Archer, Papermaster and Good, 1964; Clark, 1968; Hwang, Sugimura, Ohtaishi and Kudo, 1968; Soderstrom and Stenstrom, 1969).

This structural maturation of lymphoid tissue is paralleled by
an/

an increase in immunocompetence after 2 weeks of age. Mice cannot produce a mature antibody response to mumps virus vaccine and heterologous cells until at least 2 weeks old (Overman, 1954; Moulton and Storer, 1962). Increasing neonatal IgG synthesis (previously masked by maternal antibody) can be detected at 4 weeks of age (Fahey and Barth, 1965), and the immunological capacity of spleen cells transferred to irradiated adult recipients increases rapidly between 1 and 4 weeks of age (Makinodan and Peterson, 1962). Immunoglobulin formation becomes detectable in vitro between 10 and 21 days of age (Stecher and Thorbecke, 1967), and it is also at this age that mice born in E.coli-free conditions become resistant to oral infection with a human strain of E.coli (Mushin and Dubos, 1965). In the rat, specific cortical localisation of injected antigen, as detected by autoradiography, increases rapidly after 2 weeks of age and this is associated with a rapid increase in the ability of rats to produce antibody (Williams, 1966; Williams and Nossal, 1966). Cellular immunity is also poor in newborn mice, the ability to resist graft versus host reactions by injected adult spleen cells being delayed until some days after birth (Howard and Michie, 1962). The initial increase in lymphocytes in the villus epithelium thus appears to be associated with a morphological and functional maturation of the peripheral lymphoid tissue.

Another major physiological change taking place in 2 - 3 week old mice, is a change in the intestine itself. Round about this time the animals begin to eat solid food and cease to absorb maternal antibodies from the milk (Halliday, 1959; Hemmings and Norris, 1959). This 'closure' of the intestine to macromolecules is associated with morphological/

morphological changes and changes in the enzyme content of the absorptive epithelial cells from those characterising the neonate to those of the adult (Clark, 1959; Graney, 1968; Wissig and Graney, 1968; Clarke and Hardy, 1969; Herbst and Sunshine, 1969; Kraehenbuhl and Campiche, 1969; Williams and Beck, 1969). Thus, the increase in intestinal lymphocyte population occurs at a time when young mice begin to eat solid food and when maternal antibodies are no longer absorbed.

The simultaneous change in immunological competence and intestinal physiology suggests that some common control mechanism may be involved. Various studies support this view and suggest that the adrenal cortex may induce these changes.

Active secretory activity begins in the foetal mouse cortex on the 18 - 19th day of gestation (Eguchi, 1963). However, during the first week of life there is slow adrenal growth, the cortex especially tending to fall behind the growth of the body as a whole (Moog, Bennett and Dean, 1954). In rats there is no fall in the ascorbic acid levels during the first week even if the animal is stressed (Shapiro, Geller and Eiduson, 1962; Levine and Lewis, 1963). Beginning in the second week and continuing through the third, the mouse adrenal grows more rapidly than the body as a whole and there is a marked loss in fat and carbonyl compounds (Moog, Bennett and Dean, 1954). Similarly, in the rat, there is a fall in adrenal ascorbic acid content and the adrenal becomes stress responsive (Shapiro, Geller and Eiduson, 1962; Levine and Lewis, 1963). Thus, during the first 2 weeks of life the adrenal cortex is quiescent, and it is during this period that early lymphoid peripheralisation takes place from the thymus, and that the intestine absorbs maternal antibody.

Studies/

Studies of the effects of steroid administration further support the concept of an adrenal involvement in neonatal physiology. Administration of corticosteroids during the first few days of life appears to prevent lymphoid peripheralisation and the animals die of wasting disease (Schlesinger and Mark, 1964; Keast and Walters, 1968; Jutila, 1969). Also, closure of the intestine to macromolecules, as evidenced by an increase in alkaline phosphatase activity, occurs earlier following the administration of cortisone in the first few days of life (Moog, 1953; Halliday, 1958; Clark, 1959; Halliday, 1959; Doell and Kretchmer, 1964). Thus, circumstantial evidence favours the hypothesis that the adrenal cortex plays a major part in the maturation of both lymphoid tissue and intestine.

In summary, a study of the ontogeny of the lymphocyte population in the intestinal epithelium indicates that this population is virtually absent at birth and remains poorly developed for the first 2 weeks of life, after which there is a marked and sustained increase up to 10 weeks of age. The results do not support the hypothesis that this population is the mammalian equivalent of the bursa of Fabricius, but are consistent with the view that it is associated with the morphological maturity of peripheral lymphoid tissue, development of immunocompetence, and also with the morphological and functional maturation of the digestive tract.

B. EFFECT OF GERM-FREE LIFE AND 'CONVENTIONALIZATION'.

INTRODUCTION.

The previous section examined the ontogeny of the lymphocyte population/

population in the villus epithelium in terms of the hypothesis that this lympho-epithelial relationship constitutes the mammalian equivalent of the avian bursa of Fabricius (Fichtelius, 1968). The known central or primary lymphoid organs in vertebrates, namely the thymus and the bursa of Fabricius, appear to develop independently of antigenic stimulation, their ontogeny being similar qualitatively, and to a large extent quantitatively, in both germ-free and conventional animals (Thorbecke, Gordon, Wostman, Wagner and Reyniers, 1957; Wilson, Bealmear and Sobonya, 1965; Uno, Sumi and Sakura, 1968; Kruml, Ludvik, Trebichavsky, Mandel and Kovaru, 1969). The purpose of this study, therefore, was to examine the ontogeny of the lymphocyte population in the villus epithelium of germ-free mice and to observe the response, if any, of this population in young adult germ-free mice following exposure to a conventional environment.

MATERIALS AND METHODS.

Ontogeny.

Germ-free C3H mice of both sexes were killed at 1 - 2 days and 1, 2, 4, 6, 8 and 16 weeks of age. The proximal segment of small intestine was removed and the number of lymphocytes per 1000 epithelial cells determined for 5 sections from each of 6 mice from the different age groups.

Conventionalization.

Groups of germ-free mice were removed from the isolator at 8 - 12 weeks/

weeks of age, placed in cages contaminated with faeces from the conventional mouse colony, and reared in the ordinary animal room. Five mice were killed at 1, 2, 4, 6, 10, 14, 21 and 28 days later, and the number of lymphocytes per 1000 epithelial cells determined for 5 sections from each mouse.

RESULTS.

Ontogeny.

There was a slight increase in the number of lymphocytes during the first 2 weeks of life followed by a marked increase between the 2nd and 4th week. A further slight increase occurred up to 6 weeks, after which the number of lymphocytes remained more or less constant up to 16 weeks of age (Figure 5.2; Table 5.2).

Conventionalization.

During the first 48 hours after exposure to a conventional environment, there was a slight, but not significant, fall in the number of lymphocytes. However, from the 2nd day onward, the number of lymphocytes increased sharply up to the 6th day, and more slowly thereafter (Figure 5.3; Table 5.3).

Significant changes also occurred in the morphology of the villi, and the mesenteric lymph node. The villi of the germ-free mouse were long and slender. The lamina propria consisted of a sparse stroma containing only a few lymphocytes and mononuclear cells, and plasma cells were rare. The crypts were very shallow compared with those of the conventional/

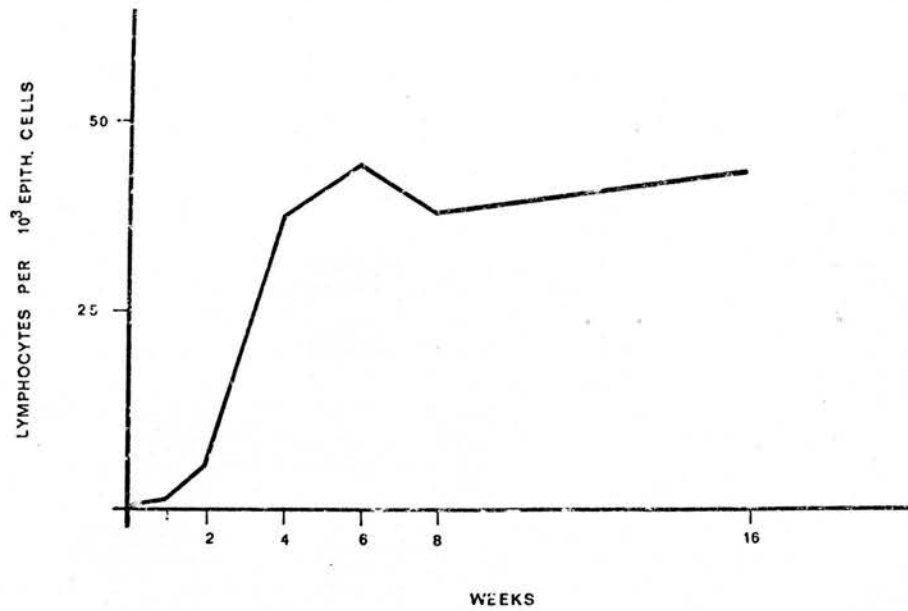


Figure 5.2 The number of lymphocytes in the intestinal epithelium of germ-free mice between birth and 16 weeks of age.

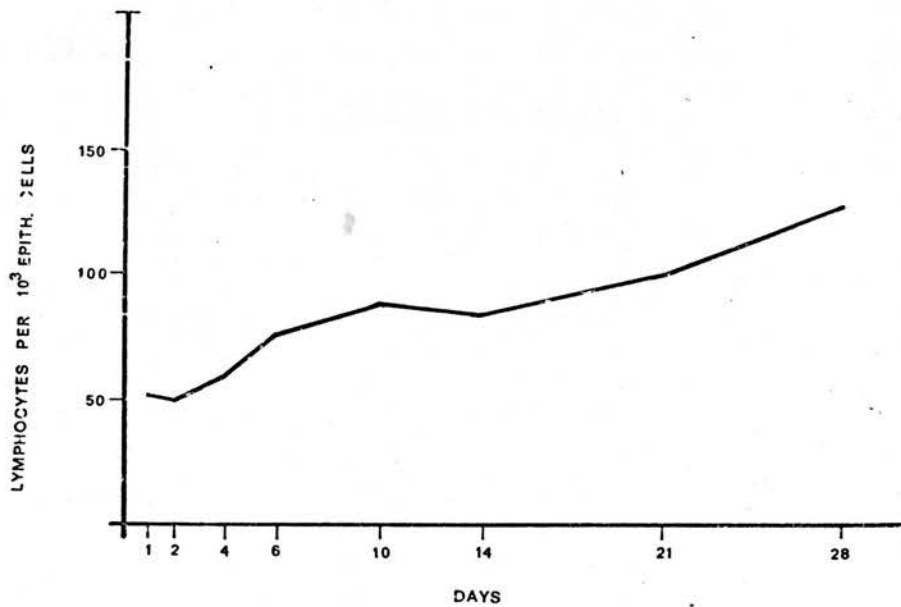


Figure 5.3 The number of lymphocytes in the intestinal epithelium of germ-free mice that have been exposed to a conventional environment for up to 4 weeks.

Plate 5.1 Section of small intestine from
a germ-free mouse. Note the
long slender villi and the sparse
cellularity of the lamina propria.
Methyl Green-Pyronin x 50 (approx.).

Plate 5.2 Section of small intestine from a
conventional mouse. The villi are
much shorter and more cellular than
those of the germ-free mouse.
Methyl Green-Pyronin x 50 (approx.).

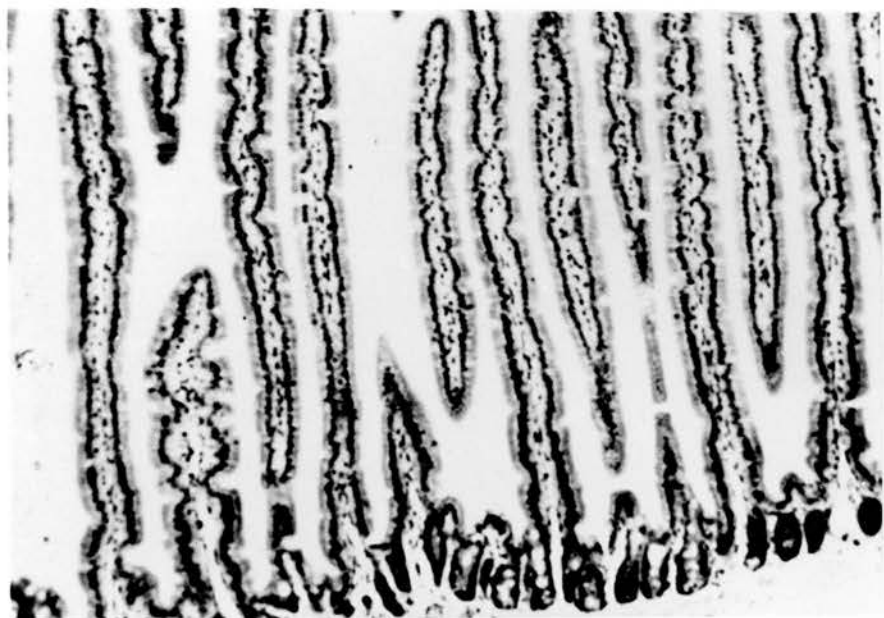


TABLE 5.2.

The number of lymphocytes per 1000 epithelial cells in sections of villi of germ-free mice at different ages.

Age	Number of lymphocytes per 1000 epithelial cells in mouse number [*]						Mean ± S.D.
	1	2	3	4	5	6	
Newborn	0.8	1.0	1.0	1.2	0.6	0.6	0.87 ± 0.2
1 week	1.2	1.6	1.4	1.0	1.2	1.2	1.26 ± 0.23
2 weeks	5.0	4.0	5.6	3.6	6.6	3.6	4.7 ± 1.7
4 weeks	35	37	36	41	41	34	37.3 ± 3.0
6 weeks	56	43	27	41	47	53	44.5 ± 10.3
8 weeks	42	42	40	35	29	37	37.5 ± 5.0
16 weeks	37	50	41	46	47	39	43.3 ± 5.0

^{*}Mean of 5 counts in non-consecutive sections.

conventional animal, and there appeared to be fewer mitotic figures.

There was little obvious change in the histology for the first 8 - 10 days, but between 10 and 21 days there was a gradual transition to the conventional morphology, with longer crypts, shorter and wider villi, and a well developed lamina propria (Plates 5.1 and 5.2).

The mesenteric lymph node of the germ-free mouse was smaller than that of the conventional mouse. On section, the cortex contained small primary nodules at the periphery, and the paracortical area lacked the cell density usually seen in conventional nodes. Germinal centres were absent and the medullary cords were only sparsely populated with lymphoid cells. Two to four days after conventionalization, pyknotic nuclei/

TABLE 5.3.

The number of lymphocytes per 1000 epithelial cells in sections of villi of germ-free mice after increasing periods of exposure to a conventional environment.

Days after exposure	Number of lymphocytes per 1000 epithelial cells in mouse number *					Mean ± S.D.
	1	2	3	4	5	
1	48	47	63	51	49	51.6 ± 6.6
2	46	43	39	52	67	49.5 ± 10.9
4	67	50	63	57	58	59.0 ± 6.4
6	60	71	88	74	89	76.4 ± 12.2
10	86	78	100	85	91	88.0 ± 8.2
14	96	91	77	75	79	83.6 ± 9.3
21	108	82	98	102	107	99.4 ± 10.5
28	110	112	136	139	144	128.2 ± 16.0

*Mean of 5 counts in non-consecutive sections.

nuclei appeared in the paracortical area. There was an increased cellularity of this area at 6 days, and simultaneously, a few germinal centres appeared. These features were well developed by 10 days. By 14 days, the ex-germ-free nodes were indistinguishable histologically from conventional mesenteric lymph nodes.

DISCUSSION.

During the first 4 weeks after birth, the ontogeny of the lymphocyte/

lymphocyte population in the villus epithelium of the germ-free mouse is similar both qualitatively and quantitatively to that of the conventional mouse. There is a slow rise during the first 2 weeks of life followed by a more rapid increase thereafter. However, the lymphocyte population of the conventional mouse continues to expand, whereas that of the germ-free mouse remains relatively static. Although the similarity in development during the first 4 weeks of life could be used to support the hypothesis of a bursal equivalent, the relatively late development of this lymphocyte population and the fact that beyond 4 weeks of age the development is dissimilar, argues against this hypothesis.

Since the conventional animal has certain metabolic differences from the germ-free animal (Goldsmith, 1965; Coates, 1968; Kellogg and Wostman, 1968), the difference seen in the intestinal lymphocyte population may indicate differences in the requirement for lymphocytes in this region of the gut to perform some metabolic function. For example, the lower rate of cell turnover of the intestinal epithelium found in germ-free animals (Abrams, Bauer and Sprinz, 1963; Kenworthy and Allen, 1966; Khoury, Floch and Hersh, 1969) could be reflected in a lower requirement for lymphocytes in this region, if one postulates a trephocytic function for these cells (Kelsall and Crabb, 1958). Similarly, if these cells play a role in fat absorption (Zawarykin, 1883), the differences in lymphocyte population between conventional and germ-free animals may be associated with the differences found in lipid metabolism (Kellogg and Wostman, 1968).

Although the lymphocyte population in the villus epithelium may have such a metabolic function, hypoplasia of this population in the germ-free/

free mouse is associated with a hypoplasia of the lymphoid tissues elsewhere in the body (Thorbecke, Gordon, Wostman, Wagner and Reyniers, 1957; Gordon, 1959; Miyakawa, 1959; Thorbecke, 1959; Bauer, Horowitz, Levenson and Popper, 1963), and with a much reduced level of serum immunoglobulin production (Gustafsson and Laurell, 1959; Wagner, 1959; Wostman, 1959; Ikari, 1964; Sell and Fahey, 1964; Fahey and Sell, 1965). Therefore, it is most likely that the smaller intestinal lymphocyte population reflects the lower level of immunological reactivity of the germ-free animal rather than any other physiological or metabolic differences from the conventional mouse.

The fact that the germ-free animals are not antigen-free may explain the similarity in development to conventional mice during the first few weeks of life. Germ-free animals are known to respond to antigens in the diet by the production of antibodies (Wagner, 1959; Crabbe, Nash, Bazin, Eyssen and Heremans, 1969), and it may be that the increase in the lymphocyte population during the early weeks of life represents a response to dietary antigens, and that the plateau phase indicates a state of equilibrium between the host response and a steady level of antigenic stimulation. Thus, if the lymphocytes in the intestine have an immunological function, the increase in the lymphocyte population of conventional animals with age could be explained in terms of a host response to continual stimulation by a much wider range of antigens than those experienced by the germ-free host.

This concept of an immunological function of the intestinal lymphocytes is further supported by the association between changes in this population and changes in both lymphoid tissue morphology and serum immunoglobulin/

immunoglobulin levels following conventionalization. After a short lag phase, the number of lymphocytes in the epithelium begins to increase and continues to increase in a manner similar to that seen in conventional animals. This increase is associated with hyperplasia of the lymphoid tissues, especially those associated with the intestine (Sprinz, Kundel, Dammin, Horowitz, Schneider and Formal, 1961; Carter, Einheber and Bauer, 1965), and with an increase in serum immunoglobulin (Gustafsson and Laurell, 1959; Wostman and Gordon, 1960).

In conclusion, the difference found in the lymphocyte population in the villus epithelium of the small intestine between germ-free and conventional mice does not support the hypothesis that this population is the mammalian equivalent of the bursa of Fabricius, a hypothesis which, if true, should show little difference in this population in animals housed in different environments. These differences in lymphocyte populations may reflect variations in metabolism between germ-free and conventional animals, with which these cells could be associated. However, the correlation between their level of development and the level of morphological and functional development of the peripheral lymphoid tissues is consistent with the hypothesis that the intestinal lymphocytes have an immunological function.

C. EFFECT OF ANTIBIOTICS.

INTRODUCTION.

A comparison of germ-free and conventional mice and the response of germ-free animals to conventionalization, has shown that there is/

is a correlation between the presence of a microbial flora and the number of lymphocytes in the villus epithelium. Although this relationship is not necessarily one of 'cause and effect', the intestinal flora can be modified by oral administration of antibiotics, and it is of interest therefore to see if the villus lymphocyte population is also modified as a result of oral antibiotics.

The changes in the enteric flora induced by antibiotics are very variable (McCoy, 1954; Sieburth, Jezeski, Hill and Carpenter, 1954), and the effect of a given antibiotic on a given flora is influenced by many factors including dose, frequency and duration of administration (Rivera and Sborov, 1951; Dubos, Schaedler and Stephens, 1963), species (Farrar and Kent, 1965), diet (Dubos, Schaedler and Costello, 1963; Dubos, Schaedler and Stephens, 1963) and season of the year (Campos, Hoener, Casta, Trabulsi and Pontes, 1958). However, there is no doubt that oral administration of antibiotics will modify the enteric flora in some, if variable, way and that this may be reflected, directly or indirectly, in an alteration of the 'physiology' of the host in terms of susceptibility to infection (Freter, 1956; Cooper, 1959; Dineen, 1961; Ashburner and Mushin, 1962; Bohnhoff and Miller, 1962), growth rate (Forbes, Park and Lev, 1959; Coates, 1962; Dubos, Schaedler and Costello, 1963), amino acid absorption (Vijayaraghavan, Murphy and Dunn, 1952; Carroll, Hensley, Sittler, Wilcox and Graham, 1953), gastric urease activity (Belding and Kern, 1963), bilirubin metabolism (Rivera and Sborov, 1951) and intestinal morphology.

It is the latter phenomenon that is of particular interest in these studies. The most commonly recorded findings are an enlarged caecum/

caecum (Pepper, Slinger and Motzok, 1953; Cooper, 1959; Clegg, 1962; Savage and Dubos, 1968; Khoury, Floch and Herskovic, 1969) and a reduction in weight of the small intestine (Pepper, Slinger and Motzok, 1953; Rusoff, Landagora and Hester, 1954; Braude, Coates, Davies, Harrison and Mitchell, 1955; Coates, Davies and Kon, 1955; Taylor and Harrington, 1955; Jukes, Hill and Branion, 1956; Barber, Braude, Mitchell, Roak and Rowell, 1957; Hill, Keeling and Kelly, 1957; Perdue, Spruth and Frost, 1957; Gordon, Wagner and Wostman, 1958; Lee and Moinuddin, 1958). The component responsible for the reduction in weight and thickness is less well understood, but a decrease in thickness of the muscularis (Clegg, 1962) and lamina propria with its associated reticulo-endothelial elements have been found (Jukes, Hill and Branion, 1956; Gordon, Wagner and Wostman, 1958; Wostman and Gordon, 1958; Gordon, 1960). Both an increase (Khoury, Floch and Herskovic, 1969) and a decrease in villus height (Jukes, Hill and Branion, 1956) have been reported.

In view of the changes in the enteric flora and the morphology of the small intestine following oral administration of antibiotics, the present study was undertaken to examine the effects of antibiotics on the level of development of the lymphocyte population in the villus epithelium of mice reared from 3 - 4 weeks of age on antibiotic-treated drinking water.

MATERIALS AND METHODS.

C57Bl mice of both sexes were reared from 20 - 24 days of age with drinking water containing either oxytetracycline HCl (Imperacin - Pfizer/

Pfizer) or Penicillin V (Compicillin - Abbott). The antibiotics were included at a concentration of 0.5 mg/ml water. The antibiotic solution and the bedding were changed twice weekly.

Five male and 5 female mice were killed after 3 days, and 1, 2, 3 and 4 weeks of treatment. Body, spleen and mesenteric lymph node weights were determined for each group and the proximal small intestine was removed for histology. The number of lymphocytes per 10 villus units was determined in 3 non-consecutive sections of intestine from each animal.

RESULTS.

Addition of antibiotics to the drinking water had no effect on weight gain, relative spleen and mesenteric lymph node weights or on the number of lymphocytes in the intestinal epithelium (Tables 5.4 and 5.5; Figures 5.4 and 5.5).

The only consistent feature observed was caecal enlargement. This was found in all mice treated with antibiotics for a week or longer, and in approximately half of the mice treated for 3 days.

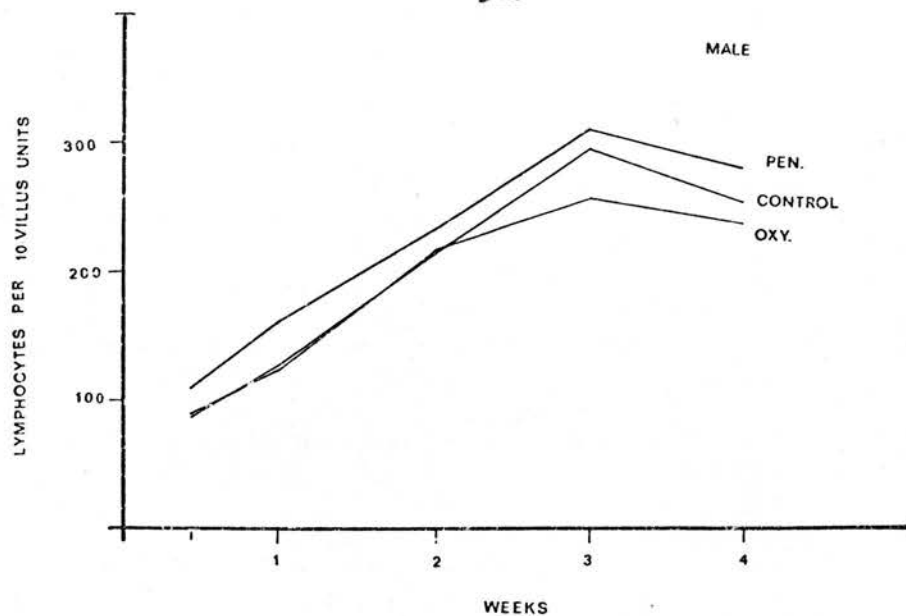


Figure 5.4 The number of lymphocytes in the intestinal epithelium of male weanling mice reared on antibiotic-treated drinking water for up to 4 weeks.

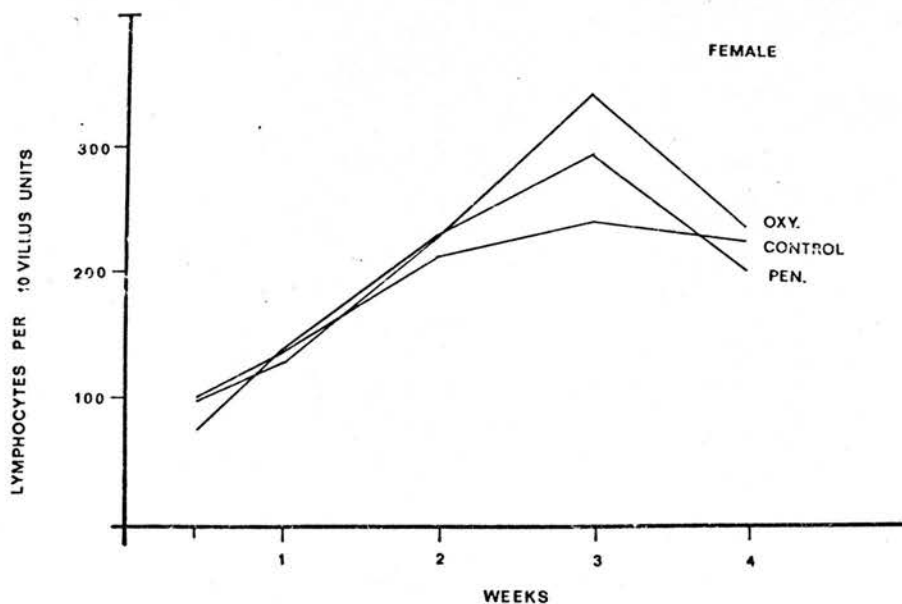


Figure 5.5 The number of lymphocytes in the intestinal epithelium of female weanling mice reared on antibiotic-treated drinking water for up to 4 weeks.

TABLE 5.4.

Changes in body weight and lymphoid tissues of C57Bl male mice given antibiotics in the drinking water from 20 - 24 days of age onwards.

Duration of treatment	Regime	Body weight (gm) \pm S.D.	Relative mesenteric L.N. weight (mg/gm B.W.) \pm S.D.	Relative spleen weight (mg/gm B.W.) \pm S.D.	Number of lymphocytes per 10 V.U. \pm S.D.
3 days	C	12.2 \pm 1.4	2.93 \pm 0.55	4.56 \pm 0.52	85 \pm 22
	P	11.3 \pm 1.3	3.53 \pm 0.44	3.17 \pm 0.77	106 \pm 38
	O	11.0 \pm 2.6	2.88 \pm 0.37	3.75 \pm 1.02	88 \pm 26
1 week	C	12.8 \pm 0.9	1.79 \pm 0.34	3.18 \pm 0.31	123 \pm 11
	P	14.7 \pm 1.9	2.08 \pm 0.23	4.57 \pm 0.86	158 \pm 38
	O	13.7 \pm 2.1	2.16 \pm 0.20	4.76 \pm 1.10	121 \pm 22
2 weeks	C	17.7 \pm 2.5	2.42 \pm 0.41	5.37 \pm 0.35	211 \pm 17
	P	19.6 \pm 1.2	2.13 \pm 0.21	4.37 \pm 0.35	234 \pm 21
	O	17.3 \pm 2.0	2.26 \pm 0.13	4.59 \pm 0.21	212 \pm 24
3 weeks	C	21.1 \pm 2.0	2.29 \pm 0.29	3.60 \pm 0.34	288 \pm 46
	P	20.0 \pm 2.0	2.82 \pm 0.35	5.13 \pm 1.09	305 \pm 30
	O	22.0 \pm 2.4	2.46 \pm 0.13	5.10 \pm 1.52	252 \pm 56
4 weeks	C	22.4 \pm 0.6	2.91 \pm 0.24	5.10 \pm 0.95	244 \pm 41
	P	24.1 \pm 1.5	2.73 \pm 0.27	3.33 \pm 0.37	274 \pm 19
	O	22.0 \pm 0.7	2.90 \pm 0.20	3.34 \pm 0.34	232 \pm 37

C = Controls. Water only.

P = Penicillin V (Compicillin) 0.5 mg/ml H₂O.

O = Oxytetracycline HCl (Imperacin) 0.5 mg/ml H₂O.

TABLE 5.5.

Changes in body weight and lymphoid tissues of C57Bl female mice given antibiotics in the drinking water from 20 - 23 days of age onwards.

Duration of treatment	Regime	Body weight (gm) \pm S.D.	Relative mesenteric L.N. weight (mg/gm B.W.) \pm S.D.	Relative spleen weight (mg/gm B.W.) \pm S.D.	Number of lymphocytes per 10 V.U. \pm S.D.
3 days	C	11.0 \pm 1.7	2.98 \pm 0.83	4.04 \pm 0.62	101 \pm 29
	P	10.6 \pm 1.1	3.51 \pm 1.15	3.42 \pm 1.09	76 \pm 16
	O	10.8 \pm 1.1	3.56 \pm 0.77	3.96 \pm 0.51	99 \pm 17
1 week	C	12.0 \pm 1.0	2.18 \pm 0.12	5.32 \pm 0.54	136 \pm 21
	P	13.8 \pm 1.0	2.37 \pm 0.15	4.38 \pm 0.72	138 \pm 29
	O	13.3 \pm 0.7	2.64 \pm 0.10	4.19 \pm 0.50	126 \pm 10
2 weeks	C	16.7 \pm 1.7	2.39 \pm 0.31	5.11 \pm 1.09	208 \pm 25
	P	16.8 \pm 1.3	2.59 \pm 0.30	5.27 \pm 0.81	226 \pm 23
	O	16.8 \pm 1.5	2.46 \pm 0.38	4.35 \pm 0.50	225 \pm 55
3 weeks	C	19.0 \pm 0.9	2.93 \pm 0.44	3.82 \pm 0.36	240 \pm 81
	P	19.2 \pm 1.4	3.79 \pm 0.22	3.71 \pm 0.34	288 \pm 53
	O	19.4 \pm 0.9	3.14 \pm 0.40	3.86 \pm 0.50	339 \pm 69
4 weeks	C	18.8 \pm 0.6	3.78 \pm 0.59	5.58 \pm 0.90	223 \pm 40
	P	19.6 \pm 0.8	4.29 \pm 0.68	4.38 \pm 0.52	198 \pm 15
	O	19.6 \pm 1.3	3.50 \pm 0.34	4.13 \pm 0.66	231 \pm 25

C = Controls. Water only.

P = Penicillin V (Compicillin) 0.5 mg/ml H₂O.

O = Oxytetracycline HCl (Imperacin) 0.5 mg/ml H₂O.

DISCUSSION.

The failure of antibiotics to influence significantly the parameters measured is not unexpected in view of the wide variation in results reported by other workers. Thus, in chickens, feeding of penicillin causes no change in the spleen weight but produces a decrease in weight and lymphocyte concentration of the ileocaecal tonsil and conversely an increase in weight and cell content of the thymus (Gordon, Wagner and Wostman, 1958). The same authors also reported that several other antibiotics, at various dosage levels, did not produce as uniform and pronounced changes as penicillin. According to Braude, Coates, Davies, Harrison and Mitchell (1955), feeding pigs with tetracyclines does not alter the spleen weight, whereas, significant differences have been noted by others (Taylor and Harrington, 1955; Barber, Braude, Mitchell, Roak and Rowell, 1957). Similarly, penicillin has been reported to cause an increase in the blood white cell counts of poultry (Glick, 1958) but in calves treated with chlortetracycline the white cell count decreases (Rusoff, Landagora and Hester, 1954). Owen and Allen (1954) and Pritchard, Riddell and Durrell (1954) found no change in the total or differential leucocyte counts in calves on antibiotic supplemented diets.

This variation in host response to oral antibiotics probably reflects the variation found in the changes seen in the gut flora resulting from such treatment. There are two main changes to be considered, changes in the absolute number of organisms and changes in the number of specific components of the population. When attempts are made to produce germ-free mice by intensive administration of antibiotics to mice housed in specially adapted boxes, the small intestinal morphology still resembles/

resembles that of conventional mice even though the bacterial count of the caecum is zero (van der Waaij, 1969). However, with lower concentrations of antibiotics, the effect on the intestinal flora is far from constant, and after an initial decrease, the absolute number of organisms often increases, even during continued antibiotic administration. This secondary increase is probably due to the proliferation of organisms which have developed resistance to the drug (Rivera and Sborov, 1951; Loh and Baker, 1955; Dineen, 1960; Hinton, 1970), or possibly due to the establishment of organisms in the intestine which are not normally able to proliferate in the presence of an indigenous flora (Freter, 1956). Thus, administration of antibiotics can lead to both qualitative and quantitative variations in the intestinal flora.

The host may respond directly or indirectly to changes in its intestinal flora. In view of the variations in flora outlined above, a direct host response will probably account for at least part of the wide disparity reported by other workers who have attempted to assess the host response in terms of body and organ weights and cell counts.

There is also the possibility of an indirect effect between flora and host in that changes in the flora may upset the balance between the host and other environmental factors. The effects of antibiotics on the host-diet relationship is probably the one section of this complex equilibrium which has received most attention. An indication of this complexity can be seen in the results of Dubos, Schaedler and Costello (1963) who found that administration of penicillin to mice reared under conventional conditions produced an increase in the rate of weight gain, whereas, mice with a defined flora showed a retardation of weight gain.

In/

In addition, the mice showed a more pronounced retardation when fed semi-synthetic diets rather than pellets. Using chlortetracycline supplementation, Vijayaraghavan, Murphy and Dunn (1952) found that the most marked stimulation of growth occurred when mice were fed on diets with critical shortage of essential amino acids. Perhaps the whole complexity of the situation is summed up in the words of Francois and Michel (1954) who concluded that 'the growth response to antibiotics is dependent on the nature of the intestinal flora which is itself a function of the dietary conditions'.

Other than the host - flora - diet interaction, little is known about other possible indirect effects of changes in flora, except that such changes may result in variations in the non-bacterial organisms in the intestine such as fungi and protozoa, to which, in turn, the host may respond (Hazen, Brown and Mason, 1953; Kligman and Lewis, 1953).

To summarize, it was not possible to produce changes in the lymphoid tissues of mice reared on antibiotic-treated drinking water, and therefore it was not possible to define the relationship between gut flora and intestinal lymphocytes which had been indicated by the response of mice to conventionalization. It is suggested that the results reflect the great potential variation in the effect of antibiotics on any given flora and thus in both the direct and indirect host responses to these changes.

CHAPTER 6.EFFECT OF DIET.INTRODUCTION.

Since the intestinal epithelium has long been known to play a major role in the absorption and assimilation of foodstuffs, the presence of a considerable number of lymphocytes in the epithelium has led to speculation that these cells may also be involved in the process of digestion (von Davydoff, 1887; Oppel, 1899; Emerson, 1906; Ohno, 1930; Shields, 1968). Although there is little or no direct evidence to support this view, changes in either the quantity of food available or some of its components may produce changes in the lymphoid tissue not only in the intestine, but also in lymphoid tissue elsewhere in the body.

Fasting of animals results in marked changes in lymphoid tissues over a very short period of time and is characterized by a lymphopenia, and an involution of lymph nodes, splenic lymphoid tissue and the thymus (Kalmarm, 1911; Jolly, 1914; Jackson, 1925; Streicher and Emmel, 1925; Gaetano, 1928; White and Dougherty, 1947; Frank, Kumagai and Dougherty, 1953). The degree of involution of different lymphoid tissues varies, the thymus being especially sensitive (Jolly, 1914). In contrast, mesenteric lymph nodes of fasting mice begin to increase in weight after an initial period of involution (Frank, Dougherty and Kumagai, 1953).

There are fewer reported studies on the intestinal lymphoid tissue, but fasting does cause a decrease in size of the lymphoid nodules (Hofmeister, 1885; Heidenhain, 1888), and at the histological level, there are decreases in the number of plasma cells in the lamina propria of/

of the villi (Crabb and Kelsall, 1956; Kelsall and Crabb, 1958), and in the number of lymphocytes in the villus epithelium (Meador, 1948).

Although the relationship between the level of dietary intake and the size of lymphoid tissues may indicate a functional role of lymphoid tissue in the absorption and assimilation of food, the high level of adrenal cortical activity (Chowers, Einat and Feldman, 1969), and the absence of lymphoid tissue involution in adrenalectomized, fasting animals (Dougherty and Kumagai, 1951; Dougherty, 1959) suggests that stress is the major factor in this involution rather than lack of demand for cells involved in digestion and absorption.

Fat absorption has been suggested as a specific function for intestinal lymphocytes. Zawarykin (1883) and Schafer (1885) were among the earliest proponents of this hypothesis. On the basis of the amoeboid forms of the leucocytes and the presence of osmiophilic particles in their cytoplasm, they suggested that lymphocytes transport fat from the intestinal lumen and epithelium to the lacteals where it is discharged. Less direct evidence for this hypothesis has come from a variety of sources. Kittens fed on a high fat diet have much greater amounts of intestinal lymphoid tissue than kittens fed on other types of high calorie diet (Settles, 1920; Lefholz, 1923). Leucocytes are strongly attracted to various fatty substances injected into the tadpole tail, where they phagocytose the fat and transport it to the lymphatics (Clark and Clark, 1917), and olive oil injected into the mouse peritoneal cavity increases the number of mitotic figures in lymph node germinal centres (Nakahara, 1922). Finally, the decrease in lymphocytes caused by X-irradiation is correlated with a decrease in fat absorption (Mottram, Cramer and Drew, 1922)./

1922).

Thus, there is evidence of a relationship between lymphocytes and lymphoid tissues, and the presence of fat, but the hypothesis that they play a role in fat transport depends largely on the demonstration of fat in the villus lymphocytes during fat absorption. Although Zawarykin (1883) and Schafer (1885) demonstrated osmiophilic particles in lymphocytes, many of these were later shown by other workers, including Schafer, to be pseudofat droplets (Heidenhain, 1888; Schafer, 1912; Leach, 1938). Therefore, if intestinal lymphocytes do play a role in fat absorption, it is unlikely to be that of fat droplet transport as suggested in the original hypothesis of Zawarykin.

Less emphasis has been placed on the possible role of lymphocytes in protein absorption than their role in fat transport. However, Paton (1922) calculated that the volume of leucocytes flowing through the intestine is sufficient to cope with the weight of protein ingested at each meal, and there are several reports on the effect of dietary protein levels on the various body lymphoid tissues.

Feeding protein-free diets causes a leucopenia, one feature of which is a decrease in the number of lymphocytes (Wissler, 1947; Asirvadham, 1948; Guggenheim and Buechler, 1949; Aschkenasy, 1965; 1969). The lymphopenia is due mainly to a decrease in the number of medium and large lymphocytes, with a much slower rate of decrease in small lymphocytes (Aschkenasy, 1965). Protein depletion also causes a reduction in weight of the organised lymphoid tissue, characterized histologically by a decrease in size of the lymphoid follicles, a reduction of the number of cells in the medullary cords and a decrease in the number of plasma cells (Asirvadham, /

(Asirvadham, 1948; Aschkenasy, 1957; 1965; Kenney, 1969).

The peripheral blood lymphocytes and the lymphoid tissue recover when protein is reintroduced into the diet, but the rate of recovery depends upon the timing and duration of the protein restriction period (Kenney, 1969). The recovery rate also depends on the nature of the protein that is refed. Guggenheim and Buechler (1949) showed that the leucopenia produced in rats by protein deficient diets recovers faster when they are fed meat or egg powder as a protein source, rather than gelatin, wheat, or maize protein. Casein, peanut and soyabean protein occupy an intermediate position. Aschkenasy (1965; 1968) examined the recovery period in more detail and found that the medium and large lymphocyte population expand by transformation of the small lymphocytes, and that the rate of recovery is greater in rats fed on a protein they had previously experienced than in rats fed on a 'new' protein source.

The effect of protein feeding on the intestinal lymphoid tissue is less well documented. Animals on diets with a very high egg albumen content show hyperplastic reactions in the lymphoid tissue of the intestinal wall and mesenteric lymph node (Wiseman and Akeroyd, 1938), and an accumulation of large numbers of leucocytes in the mucosa of the intestine (de Winiwarter, 1930). Thus there is some evidence that changes in dietary protein induce changes in lymphoid tissues, both in the intestine and elsewhere in the body, but there is no evidence that lymphoid tissues are involved in the process of protein absorption.

Finally, feeding of carbohydrates may produce changes in lymphoid tissue. Oral administration of a single dose of glucose or dextrose produces/

produces a transient lymphopenia which is maximal 1 - 4 hours after dosing (Freeman and Elmadjian, 1946; Vebelin, 1953). The blood glucose and lymphocyte levels show an inverse relationship. Lymphopenia is not produced in glucose-fed adrenalectomized animals, suggesting that the changes in blood lymphocyte level may be a response to adrenal cortical activity (Elmadjian, Freeman and Pincus, 1946). Prolonged administration of glucose causes thymus atrophy (Vebelin, 1953).

In summary, there is some evidence that lymphoid tissue, including that in the intestine, is influenced by the quantity and quality of the diet. Although these changes may reflect fluctuations in response to functional demands for lymphoid cells during digestion, there is little evidence to support this view. In this section, some aspects of the relationship between diet and the intestinal lymphoid tissue have been re-examined with particular emphasis on the lymphocytes in the epithelium.

MATERIALS AND METHODS.

Preparation of Diets.

The composition of the semisynthetic diets is given in Table 6.1.

With the exception of the protein-free diet, the food was prepared as follows. The ingredients were measured out and mixed together in a large plastic bowl. When thoroughly mixed, a quantity of water sufficient to produce a thick dough was added. The dough was then floured and rolled out to about half an inch in thickness. The resulting slab was allowed to air dry at room temperature on a wire grill for 2 - 3 days and/

TABLE 6.1.

Composition of semisynthetic diets used to assess the effect of diet on lymphocytes in the small intestinal epithelium.

Ingredients [*]	Protein free	Casein diet	Albumen diet	40% fat Exp. 2	10% fat Exp. 2	40% fat Exp. 3	3% fat Exp. 3
Maize Starch ¹	770	570	570	190	-	170	540
Casein ¹	-	200	-	200	200	-	-
Egg Albumen ²	-	-	200	-	-	200	200
Corn Oil ³	110	110	110	-	-	-	-
Margarine ⁴	-	-	-	400	100	400	30
Cellulose ⁵	10	10	10	50	50	50	50
Salts ²	40	40	40	40	40	40	40
Vitamins ²	20	20	20	20	20	20	20
Sucrose ¹	50	50	50	100	100	100	100
Cholic Acid ¹	-	-	-	-	-	20	20

^{*}All values expressed as grams except Corn Oil (ml).

1 = British Drug Houses (B.D.H.).

2 = Nutritional Biochemical Corporation, Ohio.

Salts - Wesson salt mixture.

Vitamins - Diet fortification mixture.

3 = 'Mazola' brand - Brown and Polson, Ltd.

4 = 'Tomor' brand - Van Den Berghs, Ltd.

5 = CFl cellulose - Whatman Biochemicals.

and then broken up into suitably sized pieces. This procedure was not suitable for protein-free diets. To prepare these, all the ingredients except the starch were mixed together. Boiling water was poured onto the starch, stirring continuously, until a thick sticky paste was produced. The paste was then allowed to cool before the rest of the ingredients were mixed in and then a small amount of water was added before rolling out and drying as before.

Experiment 1:- Short-term response to fat.

Eight to nine week old C57B1 mice of both sexes were given 0.2 ml olive oil by stomach tube. Three male and 3 female mice were killed for histology 1, 2, 4 and 6 hours after dosing. Three male and 3 female untreated mice were killed at the beginning and end of the experiment. The number of lymphocytes/5 villus units was determined.

Experiment 2:- Long-term response to fat.

Eight to nine week old Ash CS1 female mice were divided into 3 groups and fed either stock diet, or semisynthetic diets containing either 10 per cent or 40 per cent margarine as a source of fat and casein as the source of protein. Eight mice from each group were killed on the 3rd, 7th and 14th day of feeding and the number of lymphocytes/5 villus units determined.

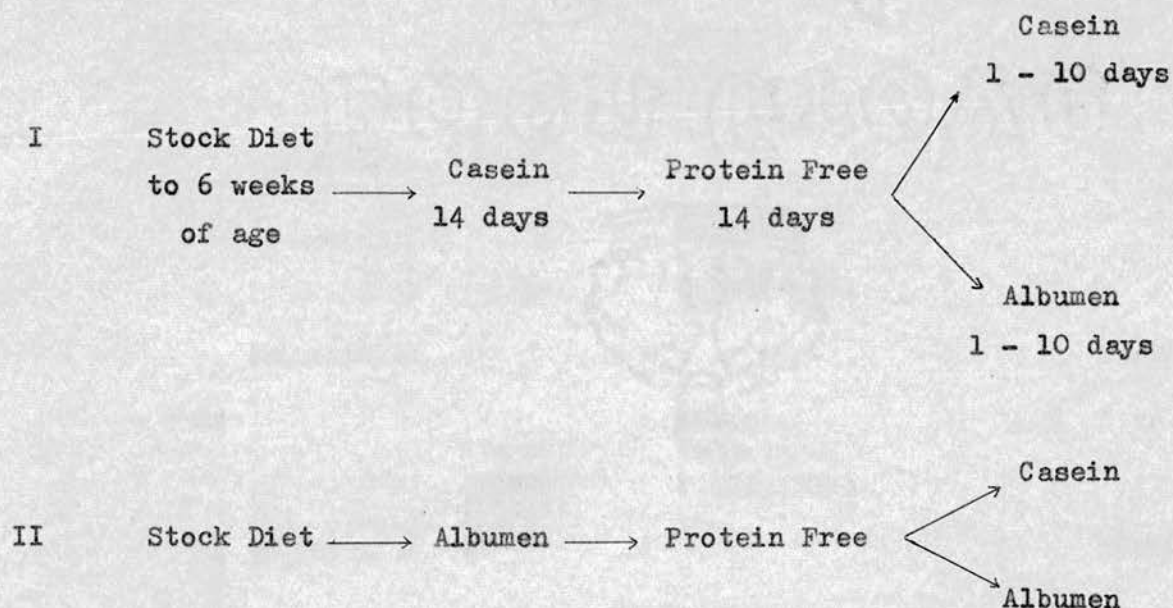
Experiment 3:- Self-induced fasting.

This experiment was originally designed as Experiment 2 but the mice ate very little of the semisynthetic diets. C57B1 mice of both sexes/

sexes were divided into 3 groups and fed either stock diet or semi-synthetic diets with 3 per cent or 40 per cent margarine as a source of fat and albumen as the source of protein. Two per cent cholic acid was also included in the diet. Five male and 5 female mice from each group were killed on the 3rd and 7th day of feeding after which some of the mice began to die and the experiment was terminated.

Experiment 4:- Effect of protein depletion and repletion.

Monocontaminated (a Clostridial sp.) C3H mice were reared on a stock diet containing neither casein nor albumen (C.D.R. diet, E. Dixon and Son, Ware). At 6 weeks of age the mice were placed on semisynthetic diets containing either 20 per cent casein or albumen as a protein source. After 14 days, the mice were placed on a protein-free diet for a further 14 days and then protein was restored to the diet, either as the original source, or in the form of a protein not previously experienced by the mice. The design is more clearly indicated by the following diagram.



RESULTS.Experiment 1:- Short-term response to fat.

Within 1 hour after administration of olive oil milky intestinal contents could be observed in the small intestine, and at 2 hours the mesenteric lymphatics were readily visible. However, there were no changes in the numbers of lymphocytes in the intestinal epithelium during the process of fat digestion (Table 6.2).

TABLE 6.2.

The effect of oral administration of olive oil on the numbers of lymphocytes in the small intestinal epithelium of 8 week old C57Bl mice.

Treatment	Time after treatment	Lymphocytes/5 V.U. \pm S.D.	
		Male	Female
None	0	129 \pm 23	127 \pm 10
0.2 ml olive oil intragastrically	1 hr.	121 \pm 20	114 \pm 1
0.2 ml olive oil intragastrically	2 hr.	127 \pm 4	125 \pm 14
0.2 ml olive oil intragastrically	4 hr.	127 \pm 22	124 \pm 20
0.2 ml olive oil intragastrically	6 hr.	143 \pm 13	133 \pm 5
None	6 hr.	148 \pm 38	140 \pm 9

Experiment 2:- Long-term response to fat.

With the exception of mice on the 10 per cent fat diet (whose cage flooded on day 12) the mice gained weight normally. Feeding mice on a high fat diet for up to 2 weeks had no effect on the number of lymphocytes in the small intestinal epithelium (Table 6.3).

TABLE 6.3.

The effect of feeding a high fat diet on the number of lymphocytes in the small intestinal epithelium of 8 week old Ash CS1 female mice.

Duration of Diet	Type of Diet	Body weight gm. \pm S.D.	Lymphocytes/ 5 V.U. \pm S.D.
3 days	Stock	23.7 \pm 1.7	200 \pm 21
	40% fat	23.6 \pm 1.6	182 \pm 16
	10% fat	23.1 \pm 1.8	202 \pm 26
1 week	Stock	25.1 \pm 1.5	191 \pm 16
	40% fat	25.6 \pm 2.0	207 \pm 42
	10% fat	25.0 \pm 2.9	211 \pm 26
2 weeks	Stock	29.1 \pm 2.2	232 \pm 46
	40% fat	28.8 \pm 1.1	231 \pm 23
	10% fat	25.7 \pm 1.9	208 \pm 38

Experiment 3:- Self-induced fasting.

C57Bl mice fed on semisynthetic diets containing albumen as the source of protein and cholic acid as an emulsifying agent ate very little of the diet provided and rapidly lost weight. The number of lymphocytes in the small intestinal epithelium also decreased (Table 6.4). Although the lymphoid organs were not weighed, it was noted that both the thymus and the spleen were much reduced in size compared with the controls.

Mice in the low fat groups began to die after 7 days and the experiment was terminated.

TABLE 6.4.

The effect of feeding diets, which were not readily eaten, on the number of lymphocytes in the small intestinal epithelium of 8 week old C57Bl mice.

Duration of Diet	Type of Diet	Body weight gm. \pm S.D.		Lymphocytes/ 5 V.U. \pm S.D.	
		Male	Female	Male	Female
3 days	Stock	22.7 \pm 1.3	18.9 \pm 0.9	144 \pm 22	148 \pm 7
	40% fat	20.6 \pm 0.8	16.6 \pm 1.1	110 \pm 12	73 \pm 3
	3% fat	18.4 \pm 1.4	16.1 \pm 1.0	75 \pm 4	74 \pm 16
1 week	Stock	24.1 \pm 1.8	19.9 \pm 0.7	116 \pm 8	148 \pm 15
	40% fat	19.5 \pm 0.9	16.8 \pm 1.2	70 \pm 8	61 \pm 16
	3% fat	18.0 \pm 0.7	14.5 \pm 1.2	50 \pm 7	54 \pm 6

Experiment 4:- Protein depletion and repletion.

The main observation in this study was that the rate of recovery of protein-depleted animals after restoration of protein to the diet was extremely variable. Some animals recovered fully in terms of regain in body, spleen and lymph node weight, others only partially recovered, and some failed to recover at all. The only common denominator in this variation was that it was generally, but not always, a cage associated phenomenon, in that animals in one cage showed similar rates of recovery. The variation was not associated with difference in sex or diet nor was it confined to any one isolator. The reason for this variation is unknown, but because it is not restricted to any diet or one sex it possibly represents a marginal condition which occurs at random. Perhaps 14 day depletion is near to the limit of the resistance of the mice or possibly it accentuated a marginal vitamin or salt deficiency in the diet.

As a result of this variation it was not possible to investigate the effect of different proteins on the rate of recovery of protein-depleted animals, and the results were therefore pooled to investigate the effect of protein depletion on the number of lymphocytes in the intestinal epithelium. The effect of restoring protein to the diet was estimated from the groups which fully or largely recovered on refeeding.

The results indicated in Table 6.5 and Figure 6.1 show that the lymphocyte population in the intestinal epithelium differed in its response from that found for the lymphoid organ weight. Following protein depletion, the relative spleen and mesenteric lymphoid weights decreased by 30 - 50 per cent, and when protein was restored to the diet they recovered rapidly at first and then more slowly. On the other hand, the number/

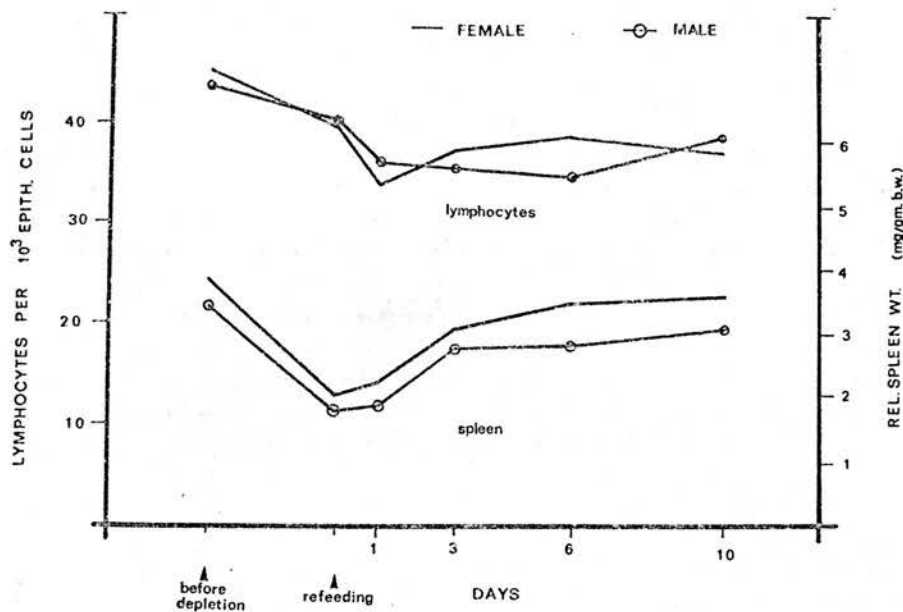


Figure 6.1 The effect of changes in dietary protein content on the number of lymphocytes in the intestinal epithelium and on the relative spleen weight.

number of lymphocytes in the intestinal epithelium decreased by only 10 per cent after protein depletion, decreased a further 10 per cent when protein was restored to the diet and only increased slightly during the period of protein repletion. Thus, in contrast to the spleen and mesenteric lymph node the lymphocytes in the epithelium show a relative resistance to protein depletion, and they only recover slightly during protein repletion, whereas the spleen and lymph node recover more or less completely.

TABLE 6.5.

Changes in body weight and lymphoid tissues of C3H monocontaminated mice after protein depletion and during protein refeeding.

Regime	Sex	No. of Mice	Body Weight gm. \pm S.D.	Relative M.L.N. Wt. mg/gm \pm S.D.	Relative Spleen Wt. mg/gm \pm S.D.	LC./10 ³ Epith. cells \pm S.D.
Before protein depletion	M	10	27.3 \pm 2.2	1.22 \pm 0.25	3.39 \pm 0.59	43.5 \pm 5.6
	F	14	22.6 \pm 1.3	1.59 \pm 0.27	3.82 \pm 0.40	45.2 \pm 4.2
After 14 days of protein depletion	M	16	19.9 \pm 2.3	0.79 \pm 0.21	1.74 \pm 0.24	39.9 \pm 5.5
	F	16	16.8 \pm 1.3	1.08 \pm 0.42	1.97 \pm 0.36	39.5 \pm 3.3
Day 1 of protein repletion	M	9	22.4 \pm 1.7	0.89 \pm 0.23	1.84 \pm 0.29	35.9 \pm 7.8
	F	10	19.5 \pm 1.0	1.05 \pm 0.32	2.15 \pm 0.20	34.0 \pm 7.3
Day 3 of protein repletion	M	11	24.1 \pm 3.2	1.22 \pm 0.19	2.71 \pm 0.17	35.3 \pm 10.3
	F	11	22.2 \pm 2.8	1.49 \pm 0.23	3.01 \pm 0.37	37.3 \pm 5.7
Day 6 of protein repletion	M	10	25.0 \pm 2.2	1.22 \pm 0.16	2.77 \pm 0.37	34.8 \pm 6.1
	F	10	22.0 \pm 2.0	1.56 \pm 0.29	3.41 \pm 0.55	39.0 \pm 9.2
Day 10 of protein repletion	M	12	27.9 \pm 3.2	1.17 \pm 0.16	3.03 \pm 0.38	38.6 \pm 6.1
	F	12	24.6 \pm 2.8	1.47 \pm 0.11	3.59 \pm 0.40	37.7 \pm 7.9

DISCUSSION.

With the exception of Experiment 4, the results would tend to agree with previous reports. Thus when mice do not eat, the decrease in size of lymphoid tissues and in the number of lymphocytes in the intestinal epithelium are similar to those which have been described for animals that have been deliberately fasted (Jackson, 1925; White and Dougherty, 1947; Meader, 1948; Frank, Kumagai and Dougherty, 1953). Since fasting has little effect on lymphoid tissues in adrenalectomized animals and since corticosteroids also produce lymphoid tissue involution, it would seem that the changes in lymphoid tissues produced by fasting are probably a stress effect.

Feeding high levels of fat in the diet or giving olive oil orally fails to influence the number of lymphocytes in the intestinal epithelium. Although these results would argue against any lymphocytic involvement in fat absorption it is still possible to postulate that lymphocytes could cope with additional fat by increasing their activity rather than their numbers. However, the evidence for fat absorption is rather tenuous, being based mainly on the demonstration of osmiophilic particles in the lymphocytes. This evidence is suspect on two grounds. First of all, many of these fat droplets have been shown to be artifacts (Heidenhain, 1888; Schafer, 1912; Leach, 1938), and secondly, it is extremely doubtful whether the resolution available with the light microscope is adequate to demonstrate that the fat droplets are actually in the cytoplasm of the lymphocytes and not in the adjacent intercellular space. This latter criticism is particularly relevant now that studies with the electron microscope have shown that transfer of chylomicrons through/

through the epithelial intercellular spaces is a major pathway during fat absorption, and lipid droplets are therefore likely to be closely adjacent to any lymphocytes which are also present in these spaces (Palay and Karlin, 1959; Ladman, Padykula and Strauss, 1963). Thus, on the evidence available, it is unlikely that lymphocytes are involved in fat absorption.

The results obtained in Experiment 4, namely, the effect of protein on the lymphocytes in the intestinal epithelium, were somewhat unexpected. The effect of protein depletion on the spleen and mesenteric lymph node, and the subsequent recovery on protein refeeding are similar to the results obtained in conventional animals (Aschkenasy, 1957; 1965; Kenney, 1969). It was expected that the number of lymphocytes in the intestinal epithelium would show a similar trend, but the results indicate that protein depletion causes only a slight decrease in cell numbers and that refeeding causes a further slight decrease after which the numbers remain more or less constant. Taking these results at face value, they do not support the hypothesis that lymphocytes in the intestinal epithelium are involved in protein digestion, a hypothesis that would expect the numbers of lymphocytes to fluctuate in tune with the variation in protein content of the diet.

To provide an alternative explanation for the observed results it is necessary to make certain assumptions. The first of these is that the lymphoid tissue involution seen in protein depleted animals is a result of stress. This may be the case since the changes in body and lymphoid tissue weights are similar to those found in animals which are fasted and in which stress is known to play a role. If involution is stress/

stress mediated it follows that the lymphocyte population in the epithelium is composed of a stress-sensitive and a stress-resistant population. This assumption is discussed more fully in the section dealing with Betamethasone activity (Chapter 7), but following the argument of Schrek and Batra (1966) it seems likely that there are differences in the steroid sensitivity of various lymphocyte populations, and this probably also holds for the intestinal lymphocyte population. Thirdly, it would appear that the germ-free or monocontaminated animal, unlike the conventional animal, has a greater proportion of stress-resistant than stress-sensitive lymphocytes. This is a rather tenuous assumption but it could be suggested that the monocontaminated animal has a lower level of antigenic stimulation in the intestine, resulting in the presence of fewer short-lived stress-sensitive effector cells. However, the effect of corticosteroids and cell labelling techniques would be necessary to confirm this point.

All these assumptions can be fitted together into the pattern of the results obtained by Aschkenasy (1965) who found that the lymphopenia caused by protein depletion was due mainly to a decrease in the number of medium and large lymphocytes, and that the small lymphocytes declined at a much slower rate. Thus, if the small lymphocytes seen in the intestinal epithelium are to a large extent stress-resistant then only a slight decrease in their numbers would be expected. When Aschkenasy examined the recovery period he found that the medium and large lymphocyte populations expand at the expense of the small lymphocyte population. Therefore, the further decline observed after protein refeeding is explicable on the basis of transformation of small lymphocytes to/

to large and medium cells which then become involved in the restoration of other lymphoid tissues of the body and do not return to the intestine.

In conclusion, it is unlikely that the lymphocytes in the intestinal epithelium are involved directly in the digestion and absorption of foodstuffs. The changes found in this population in response to changes in the quantity or quality of the diet are more easily explained by the stress concept than by attempting to correlate them with any changes in functional demand during digestion.

CHAPTER 7.EFFECT OF GLUCOCORTICOIDS.INTRODUCTION.

Lymphoid tissue is one of the more sensitive tissues to the action of corticosteroids (White, 1947; Dougherty, 1952; 1959; 1960; Dougherty, Berliner, Schneebeli and Berliner, 1964). One of the consequences is the diminished immune response and resistance to infection which follows prolonged administration of these substances (Kass and Finland, 1953; Schwartzman, 1953; Germuth, 1956; Branceni, 1968). Although the mechanism of action of corticosteroids on specific immune reactions is unknown (Sell and Asofsky, 1968), the effect on some of the tissue components and their functions in such immune reactions has been well documented. These effects include those on the thymus, peripheral lymphoid tissue, peripheral blood and thoracic duct lymphocytes and those on functional responses involving these tissues such as antibody production and delayed hypersensitivity.

The thymus and to a lesser extent the peripheral lymphoid tissues are markedly decreased in weight when large doses of adrenocorticotrophic hormone (ACTH) or corticosteroids are administered (Simpson, Li, Reinhardt and Evans, 1943; White and Dougherty, 1944; Dougherty and White, 1945; 1947; Antopol, 1950), the sensitivity of the thymus being such that it can be used as an assay for corticosteroids (Dorfman and Dorfman, 1961). The effect of corticosteroids is extremely rapid and degenerative changes can be detected in the thymus within 3 hours after injection (White and Dougherty, 1944; Ishidate and Metcalf, 1963), with the/

the maximum effect between 8 and 48 hours, depending on choice of corticosteroid, dose and duration of administration (Cowan and Sorenson, 1964; Metcalf, 1964; Claesson and Ropke, 1969; Lundin and Schelin, 1969). The degenerative changes induced involve mainly the small lymphocytes which undergo pyknosis, karyorrhexis and cytoplasmic budding while the larger lymphocytes and reticular elements survive. Oedema and active phagocytosis accompany the lymphocyte degeneration (Dougherty and White, 1945; Dougherty, Berliner and Berliner, 1961; Ishidate and Metcalf, 1963). The ultrastructural features of these degenerative changes in the thymus and lymph nodes have been described by various workers (Cowan and Sorenson, 1964; Sorger, 1968; Lundin and Schelin, 1968; 1969).

The effect of ACTH and the various corticosteroids on organised lymphoid tissue are reflected by changes in the numbers of circulating lymphocytes. The blood lymphocytes show the most dramatic response, and the lymphopenias which can be produced within a few hours may persist for several days (Dougherty and White, 1944; Reinhardt, Aron and Li, 1944; White and Dougherty, 1945; Quittner, Wald, Sussman and Antopol, 1951; Nelson, Sandberg, Palmer and Tyler, 1952). Effects on the thoracic duct lymphocytes are more variable. ACTH appears to decrease the number of lymphocytes in the thoracic duct lymph (Reinhardt and Li, 1945; Yoffey, Reiss and Baxter, 1946; Hungerford, Reinhardt and Li, 1952; Schnappauf and Schnappauf, 1968), whereas adrenal cortical extract or cortisone acetate have little effect (Valentine, Craddock and Lawrence, 1948; Hungerford, Reinhardt and Li, 1952). The effect of ACTH on the numbers of thoracic duct lymphocytes may be mediated through epinephrine rather than the corticosteroids/

corticosteroids (Hungerford, Reinhardt and Li, 1952), so that decreased numbers may indicate an interference with the circulation of lymphocytes rather than a destructive effect (Schnappauf and Schnappauf, 1968).

Corticosteroids produce functional changes in lymphoid tissues, a finding not unexpected in view of the profound morphological changes seen, immunological impairment being observed in both humoral and cell-mediated reactions. The effect of corticosteroids on antibody production is variable, depending on the type and dose of antigen, the dose of corticosteroid, and the timing of steroid injection in relation to antigen injection (Berglund, 1956). Corticosteroids given before and during antigen administration appear to have the greatest effect on subsequent primary antibody production, the suppression being most marked during the log phase of production (Berglund, 1956a; 1956b; Fragraeus and Berglund, 1961; Cox, 1968; Dukor and Dietrich, 1968; Elliot and St. C. Sinclair, 1968). Corticosteroids given after primary antigen injection have little effect on the log phase of production, but will depress the later phases of antibody production (Dukor and Dietrich, 1967; 1968; Elliot and St. C. Sinclair, 1968). These suppressive effects of corticosteroids have been attributed to an interference with antigen handling (Dukor and Dietrich, 1968), although Gell and Hinde (1953) reported little effect on phagocytosis by macrophages. In contrast to the suppressive effect on primary antibody production, corticosteroids appear to enhance the secondary response both in vivo (Dougherty, Chase and White, 1945) and in vitro (Halliday and Garvey, 1964a; 1964b).

Corticosteroids are less effective in suppressing cell-mediated reactions than antibody production (Dukor and Dietrich, 1967). Suppression of/

of homograft rejection shows a marked species variation. The graft survival time is only slightly prolonged in the mouse (Zunker and Azar, 1965; Dukor and Dietrich, 1967) in contrast to the increased survival time in rabbits (Billingham, Krohn and Medawar, 1951; Krohn, 1954), hamster (Billingham and Hildeman, 1958; Elkins, 1965) and goldfish (Levy, 1963). The immunosuppression by cortisone is more effective in animals which secrete corticosterone from the adrenal cortex than those secreting cortisol. The hamster appears to be an exception (Medawar, 1959). Although there are marked individual variations, the administration of corticosteroids either locally or systemically generally reduces or abolishes the response of sensitized humans to intradermal tuberculin (Long and Favour, 1950; Lovell, Goodman, Hudson, Armitage and Pickering, 1953; Citron and Scadding, 1957; Magnusson, 1963). Hydrocortisone appears to be more effective than cortisone (Goldman, Preston, Rockwell and Baskett, 1952). Similarly, corticosteroids abolish or inhibit the tuberculin reaction in guinea-pigs, but only when very large doses are used (Derbes, Dent, Weaver and Vaughan, 1950; Sheldon, Cummings and Evans, 1950; Gell and Hinde, 1951; Osgood and Favour, 1951; Long and Spensley, 1954). Guinea-pigs also show diminished contact sensitivity reactions when the sites are pretreated with various corticosteroids (Scott, 1965), but other workers have been unable to confirm these findings (Heald and Ridgeway, 1969).

Although the effects of corticosteroids on both lymphoid tissue morphology and function have been well documented, the precise mechanism of action of these compounds is not known. There is little doubt that steroids are taken up very rapidly by lymphoid tissues (Kellner, Dougherty and/

and Berliner, 1960), and specific receptors in thymocyte nuclei have been postulated (Brunkhorst, 1969; Lang and Stevens, 1970). Studies using cortisol indicate that although the steroids are rapidly metabolized, they start a sequence of events which are no longer dependent on the continued presence of the hormone in the gland (Kellner, Dougherty and Berliner, 1960; Bellamy, Janssens and Leonard, 1966). The events in the nucleus are somewhat obscure, but they may involve a dissociation of nuclear chromatin following the formation of phosphate-rich chromatin dispersing agents (Kleinstein, Allfrey and Mirsky, 1966; Whitfield, Perris and Youdale, 1968), with subsequent excessive gene activation and consequently, the cell literally burns itself out. One important aspect of this hypothesis is that it suggests that inactive lymphocytes with their greater nucleic acid 'protection' by histones would be more resistant to the effect of corticosteroids than rapidly dividing populations in which gene de-repression is much greater. This suggestion is borne out by the relative resistance of thoracic duct lymphocytes, and the relative sensitivity of the dividing short-lived lymphocytes to corticosteroids (Valentine, Craddock and Lawrence, 1948; Hungerford, Reinhardt and Li, 1952; Schrek and Batra, 1966; Craddock, Winkelstein, Matsuyuki and Lawrence, 1967; Miller and Cole, 1967; Esteban, 1968).

Whatever the detailed effects of corticosteroids on lymphoid tissues are, there is no doubt that they influence both structure and function. The present study was undertaken to investigate the effect of these substances on the lymphocyte population in the villus epithelium of neonate and weanling mice.

MATERIALS AND METHODS.Experiment 1:- Effect of betamethasone on neonates.

One week old C3H mice were injected intraperitoneally with 0.03 mg betamethasone 21-phosphate (Betsolan - Glaxo) per gram body weight. Littermate controls were injected intraperitoneally with 0.05 ml saline. Mice were killed for histology at 1, 7, 14 and 21 days after injection and the number of lymphocytes per 10^3 epithelial cells determined.

The nursing mothers were given oxytetracycline HCl in their drinking water (10 mg/100 ml).

Experiment 2:- Effect of betamethasone on weanlings.

C57Bl mice 20 - 24 days old were given drinking water containing betamethasone (1 mg/100 ml). Mice were killed after 3, 7, 14, 21 and 28 days of treatment and the body, spleen, thymus and mesenteric lymph node weights determined. The number of lymphocytes per 10 villus units was calculated for 3 sections of small intestine from each animal.

RESULTS.Experiment 1:- Effect of betamethasone on neonates.

Gross assessment of mice injected with betamethasone indicated that they were slightly smaller than saline injected controls and that the coat was lighter in colour. Body and relative organ weights are presented in Tables 7.1 and 7.2. The results show that after an initial depression in relative weight of 40 - 50 per cent, there is a rapid recovery in lymphoid organ weight, and 14 days after injection lymphoid weights are equivalent/

equivalent to the controls. The spleen showed the fastest rate of recovery followed by the thymus and then the mesenteric lymph node.

Injection with betamethasone had no effect on the rate of development of the lymphocyte population in the intestinal epithelium (Figures 7.1 and 7.2).

TABLE 7.1.

Effect of a single intraperitoneal injection of betamethasone (0.03 mg/gm B.W.) at 6 - 8 days of age on the subsequent development of lymphoid tissues in C3H male mice.

	Time after injection (weeks)							
	0.14		1		2		3	
	B	S	B	S	B	S	B	S
Number of mice	9	6	9	7	8	7	11	10
Body Weight (gm) ± S.D.	3.9 ± 0.3	4.5 ± 0.6	6.3 ± 1.1	7.6 ± 1.3	6.7 ± 1.1	8.0 ± 1.5	12.0 ± 0.7	13.2 ± 1.6
Relative Mesenteric L.N. Wt. ± S.D. (mg/gm B.W.)	Too small	Too small	0.94 ± 0.16	1.27 ± 0.31	1.37 ± 0.59	1.86 ± 0.73	2.13 ± 0.43	2.41 ± 0.39
Relative Spleen Wt. ± S.D.	2.66 ± 0.49	4.24 ± 1.07	3.71 ± 0.73	5.59 ± 0.67	5.78 ± 1.85	5.70 ± 1.08	4.42 ± 1.66	4.46 ± 0.73
Relative Thymus Wt. ± S.D.	1.77 ± 0.33	4.69 ± 0.41	2.74 ± 0.79	5.24 ± 0.59	5.21 ± 1.82	4.96 ± 1.43	4.05 ± 0.56	3.69 ± 0.59
Number of L.C./10 ³ epithelial cells. ± S.D.	1.8 ± 0.8	2.3 ± 1.5	14.1 ± 4.8	9.4 ± 4.9	20.4 ± 7.9	18.6 ± 6.6	31.5 ± 9.2	29.0 ± 6.5

B = Betamethasone

S = Saline

± S. D. = Standard Deviation

TABLE 7.2.

Effect of a single intraperitoneal injection of betamethasone (0.03 mg/gm B.W.) at 6 - 8 days of age on the subsequent development of lymphoid tissues in C3H female mice.

	Time after injection (weeks)							
	0.14		1		2		3	
	B	S	B	S	B	S	B	S
Number of mice	10	11	10	9	7	7	10	7
Body Weight (gm)	3.5	4.2	6.9	7.3	6.5	7.4	9.9	12.3
\pm S.D.	\pm 0.5	\pm 0.8	\pm 0.7	\pm 0.55	\pm 1.0	\pm 1.4	\pm 1.5	\pm 1.9
Relative Mesenteric L.N. Wt. \pm S.D. (mg/gm B.W.)	Too small	Too small	0.89 \pm 0.18	1.07 \pm 0.16	1.64 \pm 0.61	2.04 \pm 0.45	2.34 \pm 0.30	2.62 \pm 0.44
Relative Spleen Wt. \pm S.D.	2.55 \pm 0.66	4.26 \pm 0.81	4.13 \pm 0.53	5.03 \pm 0.57	6.64 \pm 2.50	6.48 \pm 2.16	3.45 \pm 1.07	4.67 \pm 0.74
Relative Thymus Wt. \pm S.D.	2.27 \pm 0.33	4.35 \pm 0.45	3.49 \pm 0.54	6.33 \pm 0.85	5.47 \pm 2.15	4.95 \pm 2.36	4.37 \pm 1.02	4.45 \pm 0.57
Number of L.C./ 10 ³ epithelial cells. \pm S.D.	1.7 \pm 0.9	1.7 \pm 1.0	11.5 \pm 6.3	8.8 \pm 5.1	17.4 \pm 6.8	21.3 \pm 7.2	34.2 \pm 8.5	31.6 \pm 9.1

B = Betamethasone

S = Saline

\pm S. D. = Standard Deviation

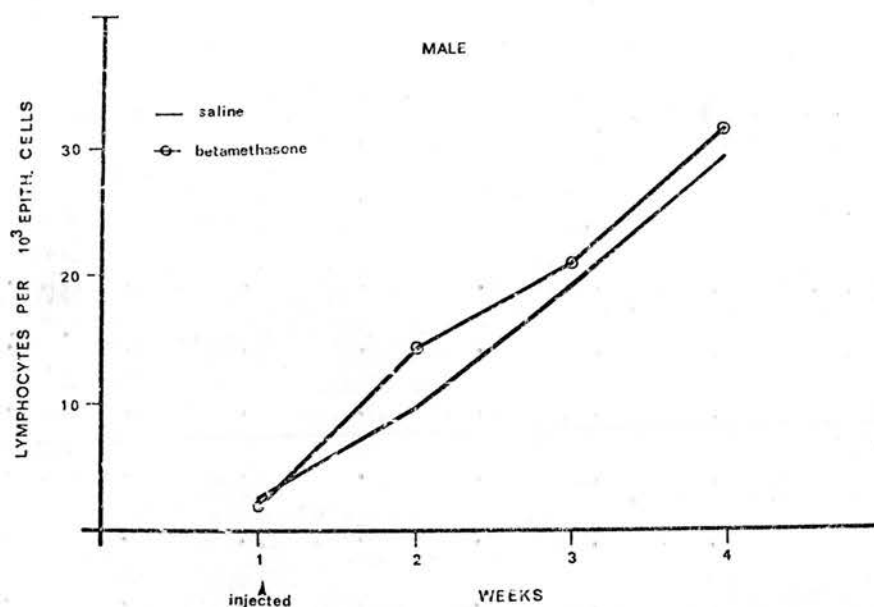


Figure 7.1 The number of lymphocytes in the intestinal epithelium of male mice killed at periods up to 3 weeks after neonatal injection of betamethasone.

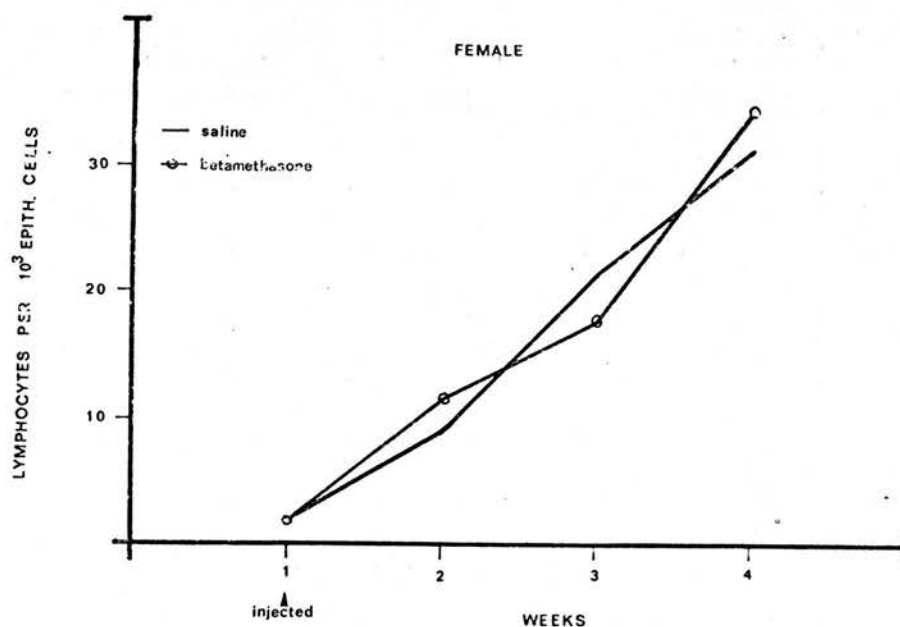


Figure 7.2 The number of lymphocytes in the intestinal epithelium of female mice killed at periods up to 3 weeks after neonatal injection of betamethasone.

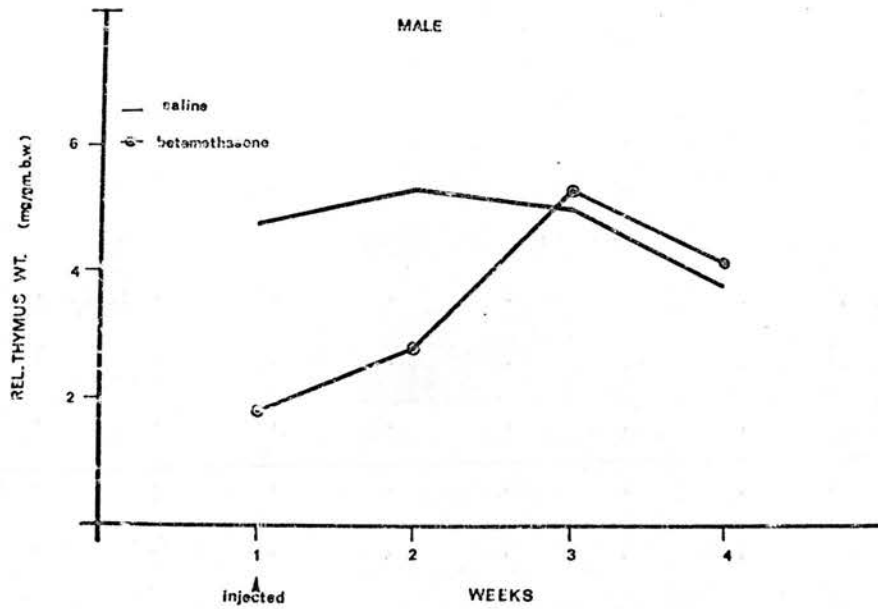


Figure 7.3 The relative thymus weight of male mice killed at periods up to 3 weeks after neonatal injection of betamethasone.

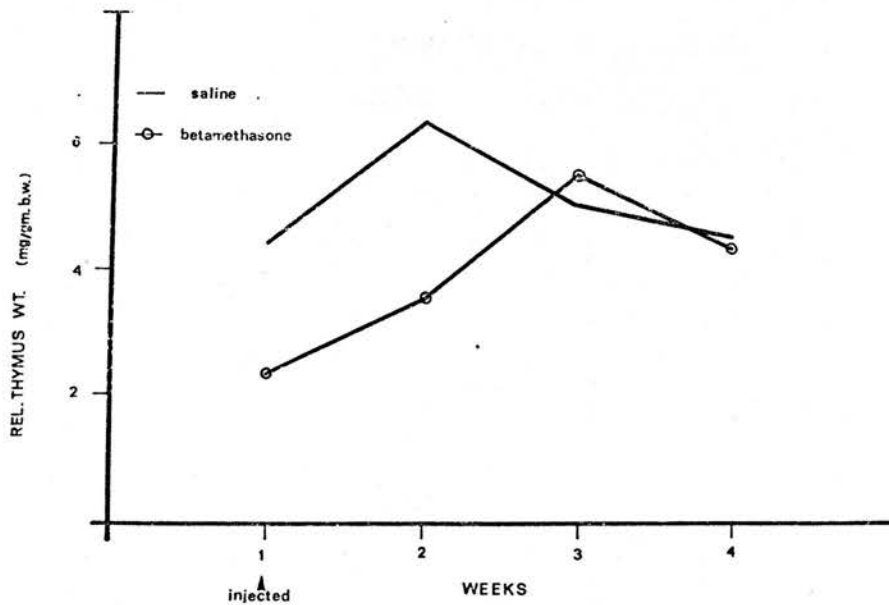


Figure 7.4 The relative thymus weight of female mice killed at periods up to 3 weeks after neonatal injection of betamethasone.

Experiment 2:- Effect of betamethasone on weanlings.

With the exception of 3 out of 5 females treated for 4 weeks with betamethasone all the treated mice survived. Treated mice failed to gain weight, whereas the controls increased their body weight by about 50 per cent. All lymphoid organ weights were markedly depressed even after 3 days of treatment. Thymus weight showed the most profound depression (Tables 7.3 and 7.4; Figure 7.6). The number of lymphocytes in the intestinal epithelium followed a trend similar to that of the lymphoid organs. The numbers were depressed after 3 days, and remained depressed during continued treatment. With the exception of the females treated for 2 weeks, the number of lymphocytes in the treated groups remained more or less constant during the period of treatment (Figure 7.5). The number of lymphocytes in the control groups increased markedly, especially between the first and third weeks of study.

During examination of sections of intestine it was noted that the villi of treated mice had a much reduced cell content of the lamina propria. In a few mice treated for 2 weeks or longer the villi were stunted in height, whereas animals from the same cage had normal villus heights. In cases with stunted villi the number of villi counted was increased to bring the epithelial cell count to approximate equivalence with that of 10 normal villus units.

The main finding in the results is that there is a rapid depression in spleen and lymph node weight, and in the numbers of lymphocytes in the intestine, after which the values remain more or less constant despite continued treatment. This does not quite hold for relative thymus weight, which does not remain constant, but shows a gradual/

gradual decline. However, untreated animals also show a decline in relative thymus weight with age, a feature characteristic of the growth curve of the mouse thymus (Axelrad and van der Gaag, 1962).

TABLE 7.3.

Changes in body weight and lymphoid tissues of C57Bl male mice given betamethasone in the drinking water (1 mg/100 ml) from 20 - 24 days of age onwards.

	Duration of treatment (weeks)									
	0.42		1		2		3		4	
	B	C	B	C	B	C	B	C	B	C
Number of mice	5	5	5	5	5	5	5	5	5	5
Body Weight (gm) ± S.D.	12.3 ± 1.7	13.2 ± 1.7	10.1 ± 0.8	13.4 ± 1.1	12.3 ± 1.2	20.9 ± 0.2	13.0 ± 1.4	20.9 ± 2.4	14.4 ± 1.1	22.5 ± 2.6
Relative Mesenteric L.N. Wt. (mg/gm B.W.) ± S.D.	1.15 ± 0.22	2.66 ± 0.31	1.61 ± 0.16	1.79 ± 0.37	1.03 ± 1.11	2.19 ± 0.38	1.21 ± 0.20	2.34 ± 0.24	1.36 ± 0.39	2.35 ± 0.56
Relative Spleen Wt. ± S.D.	1.63 ± 0.28	4.72 ± 0.28	2.02 ± 0.39	5.11 ± 1.10	2.12 ± 0.49	5.37 ± 0.61	1.90 ± 0.25	5.31 ± 0.96	1.81 ± 0.27	4.26 ± 0.83
Relative Thymus Wt. ± S.D.	0.98 ± 0.11	4.54 ± 0.30	0.55 ± 0.13	3.80 ± 0.66	0.39 ± 0.10	3.45 ± 0.35	0.37 ± 0.08	2.48 ± 0.26	0.24 ± 0.09	2.46 ± 0.49
L.C./10 V.U. ± S.D.	42 ± 17	117 ± 24	64 ± 7	103 ± 19	49 ± 30	258 ± 30	61 ± 45	201 ± 33	79 ± 22	230 ± 38

B = Betamethasone

C = Water Control

± S. D. = Standard Deviation

V. U. = Villus Units

TABLE 7.4.

Changes in body weight and lymphoid tissues of C57Bl female mice given betamethasone in the drinking water (1 mg/100 ml) from 20 - 24 days of age onwards.

	Duration of treatment (weeks)									
	0.42		1		2		3		4	
	B	C	B	C	B	C	B	C	B	C
Number of mice	5	5	5	5	5	5	5	5	7	5
Body Weight (gm) ± S.D.	12.2 ± 1.7	10.9 ± 1.9	10.3 ± 1.0	12.4 ± 0.6	12.5 ± 1.0	18.3 ± 1.5	11.3 ± 2.0	17.3 ± 1.7	12.4 ± 1.0	18.1 ± 1.1
Relative Mesenteric L.N. Wt. (mg/gm B.W.) ± S.D.	1.20 ± 0.38	2.46 ± 0.39	1.50 ± 0.23	2.29 ± 0.22	1.07 ± 0.24	2.70 ± 0.27	1.50 ± 0.22	2.87 ± 0.32	1.32 ± 0.30	2.95 ± 0.28
Relative Spleen Wt. ± S.D.	1.98 ± 0.14	4.62 ± 0.27	2.11 ± 0.31	4.98 ± 0.57	1.81 ± 0.27	4.48 ± 0.81	2.11 ± 0.66	5.00 ± 0.25	1.77 ± 0.42	5.00 ± 1.17
Relative Thymus Wt. ± S.D.	1.30 ± 0.42	4.03 ± 0.74	0.67 ± 0.10	4.87 ± 0.23	0.30 ± 0.03	3.71 ± 0.33	0.42 ± 0.09	3.50 ± 0.51	0.37 ± 0.06	4.32 ± 0.26
L.C./10 V.U. ± S.D.	77 ± 28	102 ± 31	83 ± 17	99 ± 25	20 ± 9	122 ± 30	91 ± 45	238 ± 68	91 ± 44	192 ± 78

B = Betamethasone

C = Water Control

± S. D. = Standard Deviation

V. U. = Villus Units

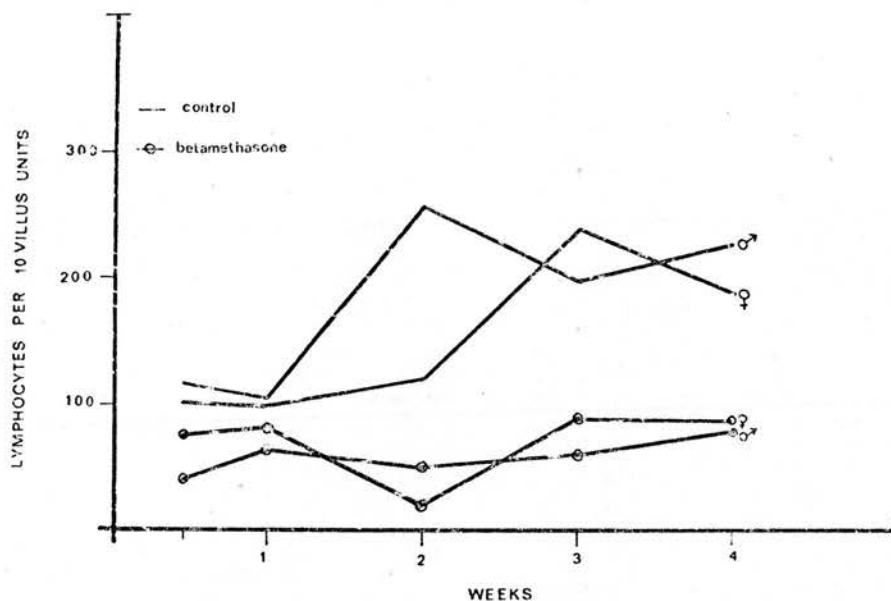


Figure 7.5 The number of lymphocytes in the intestinal epithelium of weanling mice reared for up to 4 weeks on betamethasone-treated drinking water (1 mg/100 ml).

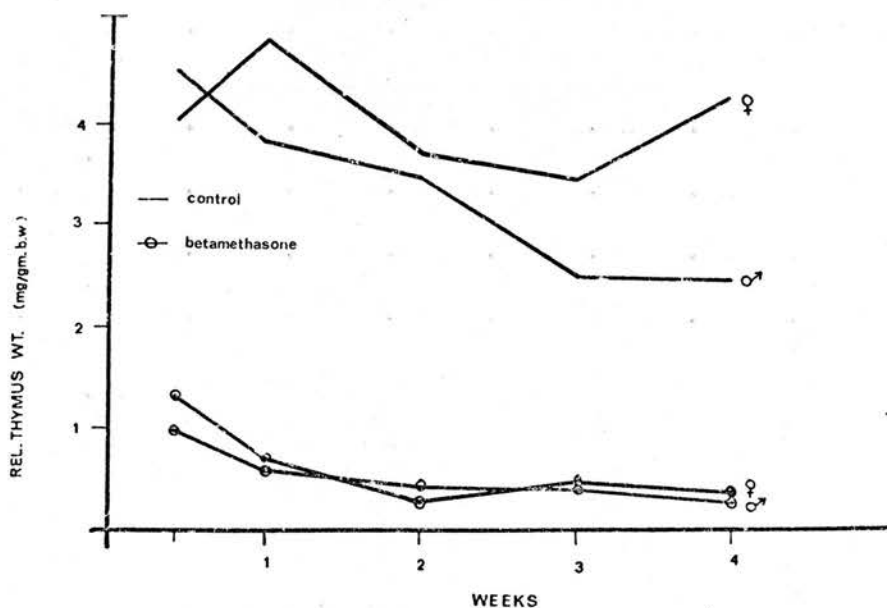


Figure 7.6 The relative thymus weight of weanling mice reared for up to 4 weeks on betamethasone-treated drinking water (1 mg/100 ml).

DISCUSSION.

The results obtained in Experiment 1 show that the administration of betamethasone at 7 days of age does not appear to have any effect on the development of the lymphocyte population in the intestinal epithelium. However, the development of the spleen, thymus and mesenteric lymph node is retarded, but only temporarily, and within 14 days after injection they have recovered more or less completely. The failure to observe any significant effect on the intestinal lymphocytes is probably due to the fact that, during the first 10 days of life especially, the numbers counted are so small ($3/10^3$ epithelial cells) that any significant alteration in numbers could easily be missed, while at the later time intervals studied the rate of increase has returned to normal. Therefore, although it is possible to conclude that betamethasone does not have any effect on the subsequent rate of development of the intestinal lymphocyte population, it cannot be concluded that corticosteroids have no effect since only very small numbers of cells are found at the critical 24 hour period in the untreated controls.

of e The effect on the thymus and peripheral lymphoid tissues agrees with the results of other workers, who found that after an initial involution there was a rapid recovery to normal levels within a few days to 3 weeks after injection (Branceni and Arnason, 1966; Clark, 1968; Jutilla, 1969). The results would therefore support the hypothesis put forward by Clark (1968), that the quiescence of the adrenal cortex in rats and mice during the first two weeks of postnatal life is a physiological mechanism which allows full development of the peripheral lymphoid tissues.

One/

One unexpected finding during this experiment was the failure of any of the treated mice to die. The only deaths recorded were due to cannibalism during the first 24 hours after injection. With the exception of Clark (1968) in mice and Branceni and Arnason (1966) in rats, the majority of other studies using conventional animals have reported severe wasting and death following neonatal corticosteroid administration (Schlesinger and Mark, 1964; Duhig, 1965; Reed and Jutilla, 1965(a); 1965(b); 1967; Keast, 1968; Keast and Walters, 1968; Salomon, Benveniste and Gueguen, 1969).

The failure to produce wasting could be due to either the dose and type of drug, or the cover provided by antibiotic treatment of the nursing mothers. The dose used in this experiment was 0.03 mg/gm body weight, whereas wasting has been reported in response to hydrocortisone or cortisone acetate given at 0.1 - 0.2 mg/gm body weight. Since betamethasone is far more potent (greater than 10X) than its parent cortisone or hydrocortisone (Atkinson, Pratt and Tomich, 1962), betamethasone was given at an equivalent or greater dose than the parent compounds. However, betamethasone is a much shorter-acting compound than the acetate derivatives of its parent compounds. Although this may explain the failure to produce wasting with betamethasone, it does not explain the results obtained by Clark (1968) who used hydrocortisone acetate and failed to produce wasting. The explanation probably lies in the type of indigenous intestinal flora present in different colonies of mice. Thus, mortality is not as common in germ-free mice (Reed and Jutilla, 1965(a); 1965(b); Salomon, Benveniste and Gueguen, 1969), or in mice reared by antibiotic treated mothers (Duhig, 1965; Reed and Jutilla, /

Jutilla, 1967). On these grounds it is postulated that the failure to produce wasting and death in this experiment was due to a combination of short-acting steroid activity and antibiotic cover during the few days of severe lymphoid tissue depletion.

Although betamethasone given as a single dose is relatively short-acting, it has the advantage that it can be administered in the drinking water over prolonged periods (Casey, 1968), and Experiment 2 was designed to examine the effects of long-term administration on the lymphocyte population in the intestinal epithelium and on the spleen, thymus and mesenteric node weights. Assuming that mice drink 5 ml of water per day, then even at the relatively low dose level used (50 µg/mouse/day) betamethasone proved extremely potent in producing lymphoid tissue involution. Marked involution was seen after 3 days of treatment, but continued treatment had no significant effect on organ weight or on the intestinal lymphocyte population. The exception was that the female mice treated for 2 weeks had an unusually low lymphocyte count for which there is no obvious explanation.

These results support the hypothesis that most lymphoid tissues in the body are composed of a cortisone-sensitive and a cortisone-resistant population. The cortisone-resistant population is probably composed of long-lived recirculating lymphocytes, whereas short-lived lymphocytes form the cortisone-sensitive population. The evidence for the resistance of the recirculating lymphocytes stems from the observations that the depletion of thoracic duct lymphocytes by corticosteroids is not due to a destructive effect, but due to an interference with their circulation (Valentine, Craddock and Lawrence, 1948; Hungerford, Reinhardt and Li, 1952; /

1952; Schnappauf and Schnappauf, 1968). Cell labelling studies also indicate that rapidly dividing cells are destroyed by corticosteroids, whereas long-lived, labelled cells persist (Miller and Cole, 1967; Esteban, 1968). Finally, in vitro studies indicate that phyto-haemagglutinin-responsive motile lymphocytes, features of long-lived populations, are also resistant to corticosteroids (Schrek and Batra, 1966).

Applying this concept to the intestinal epithelium, it would seem that the effect of oral administration of steroids is to deplete the epithelium of short-lived lymphocytes leaving a population composed largely of long-lived cells. That certain mice also show stunting of the villi may indicate that the short-lived population play a role in the cellular defence mechanism of the intestine. However, the immunological impairment produced by corticosteroids also includes antibody production, as evidenced by the depletion of plasma cells in the lamina propria, and therefore it is not possible to cite the correlation between lymphocyte depletion and stunting of villi, to support with any conviction, the concept of a cellular defence mechanism as a major function of lymphocytes in the epithelium.

In summary, administration of a single high dose of a short-acting corticosteroid to neonatal mice has little effect on the ontogeny of the intestinal lymphocyte population and only a temporary effect on other lymphoid tissues. Long-term administration to older mice indicates that the population of lymphocytes in the intestinal epithelium is composed of a cortisone-resistant and a cortisone-sensitive population, and therefore by analogy with other studies, a long-lived and a short-lived population.

CHAPTER 8.INVESTIGATIONS INTO THE HYPOTHESIS THAT LYMPHOCYTES
IN THE INTESTINAL EPITHELIUM ARE INVOLVED IN CELL-
MEDIATED (DELAYED-TYPE) HYPERSENSITIVITY REACTIONS.INTRODUCTION.

Since the early classical studies of Landsteiner and Jacobs (1935; 1936(a); 1936(b)), it has now been conclusively shown that lymphoid tissues play an integral part in cell-mediated hypersensitivity reactions. That the intestinal lymphoid tissue, and particularly the lymphocytes in the epithelium, may be involved in such reactions may be suspected for two reasons. First, the close proximity of a wide variety of antigens in the intestinal lumen, and second, both long-lived and short-lived lymphocyte populations, each of which is found in the epithelium, are involved in these reactions. Therefore, it was the purpose of this study to investigate this hypothesis further by exposing the intestine to known skin sensitizing agents and using contact hypersensitivity as a model for delayed-type reactions. The term, known skin sensitizing agents has been deliberately used since the majority of published work has involved the skin, particularly that of the guinea-pig, as an experimental model to study contact hypersensitivity.

The rest of this introduction is devoted largely to discussing current views on the skin model, followed by a short review of the published attempts to use the intestine and its associated lymphoid tissues as a similar model.

Contact sensitivity in the skin can be divided into three basic components, the afferent limb, the central component and the efferent limb./

limb. Following the application of a sensitizer to the skin, some enters the venous blood fairly rapidly, but the rest remains complexed to skin proteins (Eisen and Tabachnik, 1958). It is the sensitizer retained in the skin which is thought to be the sensitizing fraction (Macher and Chase, 1969), for extracts of skin from guinea-pigs painted with sensitizer produce sensitivity when injected into normal guinea-pigs (Parker and Turk, 1970(a); 1970(b)). The antigen-sensitive cells are thought to be small lymphocytes, many of which can be seen in normal skin (Andrew and Andrew, 1949), and the reaction of these cells with the sensitizer-protein complex probably forms the first part of the afferent limb. Lymphatic integrity is essential for sensitization (Frey and Wenk, 1957), and it is probably by this route that lymphocytes, and possibly the sensitizing complex, enter the drainage lymph node from the periphery where the central component of the reaction takes place.

The central component of the reaction is characterized morphologically by a rapid increase in the number of large pyroninophilic cells in the node (Oort and Turk, 1965; Turk and Heather, 1965; de Petris, Karlsbad, Pernis and Turk, 1966; Wyllie, 1966; Verrier-Jones and Olson, 1969; Wiest and Jung, 1970). These pyroninophilic cells actively synthesize DNA and then divide to form small lymphocytes, many of which leave the node (Turk and Stone, 1963; Oort and Turk, 1965; Diengdoh and Turk, 1966; Verrier-Jones and Olson, 1969). A marked increase in protein synthesis in the node accompanies these changes (Wilson and Turk, 1968). This proliferative phase of the lymph node response appears to be thymus-dependent since it occurs in the paracortical areas of the node (Oort and Turk, 1965), and is accompanied by/

by selective migration of thymus-derived cells to the activated node (Linna, 1970).

After a suitable interval, usually five days or more, a second application of sensitizer will evoke the efferent limb of the response. The characteristic features are, macroscopically, erythema, oedema and induration, and microscopically, pronounced mononuclear round cell infiltration (Fisher and Cooke, 1958; Waksman, 1960; Flax and Caulfield, 1963; Groth, 1964; Turk, Heather and Diengdoh, 1966; Carr, Scarpell and Greider, 1968). The response is maximal 24 to 48 hours after application. The precise composition of the mononuclear cell infiltration is difficult to determine, but histochemical studies indicate that the majority of the infiltrating cells are lymphoid cells as distinct from monocytes and macrophages (Turk, Heather and Diengdoh, 1966).

Knowledge of the pathogenesis of these challenge responses has been derived to some extent from studies of other forms of delayed hypersensitivity, particular tuberculin-type reactions, but in the absence of evidence to the contrary, it must be assumed that these findings also hold for contact hypersensitivity. Passive transfer of tritium-labelled cells from animals sensitized to one allergen to animals actively sensitized to a different allergen, followed by skin challenge of the recipient with both allergens, indicates that the infiltration of cells into the lesions is largely non-specific (Turk, 1962; McCluskey, Benacerraf and McCluskey, 1963; Turk and Oort, 1963). Using passive transfer of cells and local labelling techniques, it has been shown that this non-specific population is derived from rapidly dividing precursors located in the bone marrow (Kosunen, Waksman, Flax and Tihen, 1963; McCluskey, /

McCluskey, Benacerraf and McCluskey, 1963; Liden, 1967; Lubaroff and Waksman, 1968(a); 1968(b); Liden and Linna, 1969). However, the specificity of the reaction probably lies in a different population of cells since transfused sensitized marrow cells only migrate satisfactorily to tuberculin lesions provided that sensitized lymph node cells are also injected (Spector and Willoughby, 1968). Similar studies using transfusions of bone marrow and thymus cells show that there is an early accumulation of thymus-derived cells in the lesion which may account for the specificity of the reaction (Williams and Waksman, 1969).

In vitro studies support the suggestion that the initial stage of the reaction comprises an interaction between a population of specifically sensitized lymphocytes and specific antigen, resulting in the release of one or more factors which induce the accumulation of a non-specific population and full development of the lesion (Bloom and Bennet, 1968; David, 1968; Ruddle and Waksman, 1968; Heise and Weiser, 1969; Krejci, Pekarek, Johanovsky and Svejcar, 1969; Ward, Remold and David, 1969; Willoughby, 1969).

Although most of the investigations into contact sensitivity outlined above have used the guinea-pig skin as a model, attention is now being turned to studying contact sensitivity in other species. Of these species, with the obvious exception of man, the mouse has probably been the subject of the most intense investigation. The results obtained indicate that the same basic pattern of response occurs as in the guinea-pig, namely, following application of the sensitizer there is a proliferation of pyroninophilic cells in the drainage lymph node, and on subsequent exposure of the skin to sensitizer a response is produced which is/

is characterized by erythema, swelling and cellular infiltration (Crowle and Crowle, 1961; Asherson and Ptak, 1968; Davies, Carter, Leuchars and Wallis, 1969; de Sousa and Parrott, 1969; Dietrich and Hess, 1970). The two major variations from the guinea-pig model are the proliferation of germinal centres in the lymph node following the pyroninophilic phase (Davies, Carter, Leuchars and Wallis, 1969; de Sousa and Parrott, 1969), and the prominence of neutrophils in the skin response to challenge (Asherson and Ptak, 1968; de Sousa and Parrott, 1969).

Unfortunately, the success achieved in producing skin sensitivity in different species has not been matched by studies attempting to produce sensitivity in surface epithelia other than the skin. These latter investigations have been designed either to assess skin responses after initial epithelial application of sensitizers, or conversely, to assess epithelial responses after initial skin sensitization. The intestinal epithelium has been the stimulated surface in most of these studies.

The effect of oral administration of contact sensitizers was first reported by Chase (1946), who found that 2, 4-dinitrochlorobenzene (DNCB), administered at frequent intervals, produced a state of tolerance. Guinea-pigs so treated failed to become sensitized, as assessed by skin testing, and furthermore, were refractory to subsequent attempts at sensitization by the more usual skin route, except when the most severe sensitizing techniques were used (Battisto and Chase, 1955). These results have since been confirmed by several workers using different contact sensitizers and different dosing regimes (Coe and Salvin, 1965; Pomeranz and Norman, 1966). Similar results have also been obtained in the/

the mouse (Asherson and Ptak, 1970). Although relatively large doses of sensitizers were administered orally in these experiments (e.g. 50 mg DNCB to guinea-pigs), it has been suggested that only a very small fraction of the administered dose actually crosses the mucosal barrier, and that the tolerance produced is probably low zone tolerance (Macher and Chase, 1969).

In contrast, recent studies in other species have suggested that it is possible to induce sensitivity, rather than tolerance, by oral administration of contact sensitizers. Bicks, Azar and Rosenberg (1967) found that oral dosing of miniature swine with capsules of DNCB in an inert paste over a period of some months produced a state of skin sensitivity. Lowney (1968) attempted to produce immunological tolerance to DNCB in man by application of DNCB to the buccal mucosa, and found that although many subjects showed varying degrees of refractoriness to subsequent skin sensitizing regimes, a few did, in fact, become sensitized as a result of the initial mucosal applications. It is interesting to note that in this work the subjects were made to take a few sips of water following the mucosal application so that a weak solution of DNCB would have been swallowed.

The alternative avenue of investigation has been to challenge epithelial surfaces after the animal has been skin sensitized, mainly in an attempt to produce an experimental model of certain human gastrointestinal diseases such as ulcerative colitis and coeliac disease, in which delayed hypersensitivity reactions are thought to play a role (Kraft and Kirsner, 1966; Stewart, 1968). Using the colon of skin sensitized guinea-pigs as the target site, lesions characterized by oedema, /

oedema, congestion and mononuclear cell infiltration can be produced by rectal instillation of DNCB in an inert paste or olive oil (Bicks and Rosenberg, 1964; Rosenberg and Fischer, 1964; Bicks, Brown, Hickey and Rosenberg, 1965; Bicks, Azar, Rosenberg, Dunham and Luther, 1967). Similar results have been reported for the vagina (Macher and Dorner, 1966). However, attempts to produce contact sensitivity reactions in the small intestine have not been reported, except by Bicks, Azar and Rosenberg (1967) who produced an acute fibrino-purulent reaction in the intestine of sensitized miniature swine by administering capsules of DNCB over a period of some months.

To sum up, lymphocytes in the skin play a role in both the initiation of contact sensitivity, and in the manifestation of this sensitivity following skin challenge. In this study, an attempt was made to produce contact sensitization and challenge responses in the intestine in order to investigate the hypothesis that the lymphocytes in the intestinal epithelium can play a similar role. Demonstration of such reactivity could then be used to explain the presence of a lymphocyte population in the intestinal epithelium of the normal animal.

MATERIALS AND METHODS.

Random bred young adult guinea-pigs of both sexes and Ash CS1 (or CFW where stated) mice 8 - 10 weeks old were used as experimental animals. Both species had white coats.

Five main groups of experiments were undertaken -
GROUP A experiments were concerned with skin sensitization and skin challenge, /

challenge, i.e. standardizing skin responses.

GROUP B experiments studied the effect of oral administration of sensitizers on skin reactivity.

GROUP C experiments examined the effects of challenging the gut mucosa of skin sensitized animals.

GROUP D experiments studied the effect of a second oral administration on the intestine following initial primary doses.

GROUP E experiments were concerned with studying the mesenteric lymph node after oral administration of sensitizers in the hope of identifying a central pyroninophilic reaction.

GROUP A:- Skin sensitization.

(a) Guinea-pig skin reactions. This technique was the standard procedure used by Unilever Research Laboratories as a reference test for potential skin sensitizers. Shaved guinea-pigs were sensitized to DNCB by 4 intradermal injections of 0.1 ml 0.025 per cent DNCB in 2 per cent ethanol, one injection near each of the four axial lymph nodes. The animals were skin tested 8 days later by intradermal injection of 0.1 ml 0.01 per cent DNCB in one flank and topical application of 0.1 ml 0.1 per cent DNCB on the opposite flank. The challenge sites were recorded 24 hours later. Skin tests were repeated after a further 7 days. Positive reactors were animals with erythematous lesions greater than 9 x 9 mm at the intradermal site and diffuse or multifocal erythema at the site of topical application.

(b) Skin reactions in mice. Oxazolone (4-ethoxymethylene-2-phenyl oxazolone, B.D.H., Poole) was used as the sensitizer. Two different sensitizing regimes were tried and in one regime 3 different vehicles were tested./

tested. In all cases skin challenge was carried out 10 days later by painting both sides of the left ear with 1 per cent oxazolone in olive oil and assessing the response 24 hours later by measuring the ear thickness at 2 sites near the periphery with a micrometer (Quicktest AO2T, Carobronze Ltd., London).

Experiment A.1.

Skin sensitization was attempted by painting one ear and one front foot with approximately 0.02 ml 10 per cent oxazolone in absolute alcohol. The skin adjacent to each site was shaved prior to application of the oxazolone.

Experiment A.2.

Skin sensitization was attempted by the method of Dietrich and Hess (1970). 0.1 ml 10 per cent oxazolone in absolute alcohol was applied once daily for 3 consecutive days to the clipped abdomen.

Experiment A.3.

Experiment A.2 was repeated using absolute alcohol, acetone or olive oil as the vehicle.

Five mice were used in each group.

GROUP B:- Skin responsiveness following oral administration.

Experiment B.1.

DNCB was administered in the form of an emulsion to 20 guinea-pigs. To prepare the emulsion, 0.75 g sodium taurocholate were dissolved in 125 ml water. 250 mg DNCB were dissolved in a few drops of acetone which was then mixed with 125 ml olive oil. The DNCB/olive oil was added to/

to the sodium taurocholate solution and the complete mixture emulsified in a Waring blender for 10 minutes. Each guinea-pig was given a single oral dose of 1.5 ml emulsion (1.5 mg DNCB) for 5 consecutive days by means of a polyethylene catheter. To attempt to prevent contact of the emulsion with the oral mucosa, the emulsion was withdrawn slightly from the end of the catheter, and the end of the catheter carefully wiped before attempting gastric intubation. After administration of the required dose, the syringe plunger was again withdrawn slightly before the catheter was removed. Fifteen days after the last oral administration the guinea-pigs were skin tested, and the sites evaluated 24 hours later.

Experiment B.2.

Four groups of 15 mice were dosed by gastric intubation as follows:-

- Group 1 - 0.1 ml 0.01% oxazolone in olive oil once daily for 5 days.
- Group 2 - 0.1 ml 0.1% oxazolone in olive oil once daily for 5 days.
- Group 3 - 0.1 ml 1.0% oxazolone in olive oil once daily for 5 days.
- Group 4 - 0.1 ml 10% oxazolone in olive oil once daily for 3 days (dosing was not continued for days 4 and 5 because of the occurrence of deaths after the 3rd dose).
- Group 5 - 5 mice skin sensitized by 0.1 ml 10% oxazolone in absolute alcohol for 3 days (Positive control group).

Ten days after the last treatment all mice were ear-tested. After reading the ear test the mice were then skin sensitized and given a second ear test a further 10 days later.

Experiment B.3.

Experiment/

Experiment B.2 was repeated with the exception that the top dose group (10 per cent) was omitted, and that skin sensitization was carried out using acetone rather than alcohol as the solvent.

Experiment B.4.

Four groups of 15 mice were given 3 oral doses of oxazolone spaced 3 hours apart. The dose levels were as follows:-

Group 1 - 0.1 ml 0.01% oxazolone in olive oil.

Group 2 - 0.1 ml 0.1% oxazolone in olive oil.

Group 3 - 0.1 ml 1.0% oxazolone in olive oil.

Group 4 - 0.1 ml 10% oxazolone in olive oil.

A group of 5 animals formed a skin sensitized positive control. Ten days after treatment the mice were ear-tested, following which they were skin sensitized and given a second ear test a further 10 days later.

Experiment B.5.

Female mice were divided into 7 groups and treated as follows:-

Group 1 - 11 mice. 0.1 ml 0.1% oxazolone/alcohol intragastrically.

Group 2 - 11 mice. 0.1 ml 1.0% oxazolone/alcohol intragastrically.

Group 3 - 15 mice. 0.1 ml 10.0% oxazolone/alcohol intragastrically.

Group 4 - 11 mice. 0.1 ml 0.1% oxazolone/acetone intragastrically.

Group 5 - 11 mice. 0.1 ml 1.0% oxazolone/acetone intragastrically.

Group 6 - 15 mice. 0.1 ml 10.0% oxazolone/acetone intragastrically.

Group 7 - 5 mice. Skin sensitized positive controls.

Two days later, groups 1 and 2 were redosed, groups 4 and 5 were given a further half dose and groups 3 and 6 were given no second dose. Ten days later, surviving mice were ear-tested following which they were skin sensitized/

sensitized and then given a second ear test a further 10 days later.

Experiment B.6.

In the course of Experiment B.4 it was noted that some mice, particularly those where acetone was used as the solvent, developed erythema of the ears during the course of skin sensitization after the first ear test. This experiment was designed to investigate the possibility that oral administration 'primed' the mice to respond to sensitizer retained in the skin following the first ear test. Three groups of 15 female mice were treated as follows:-

Group 1 - 0.1 ml 0.1% oxazolone in acetone intragastrically.

Group 2 - 0.1 ml 1.0% oxazolone in acetone intragastrically.

Group 3 - 0.1 ml acetone intragastrically.

The groups were given another half dose (0.05 ml) 2 days later. Ten days after the second oral dose the mice were ear-tested and then 48, 72 and 96 hours after ear painting, the mice were skin painted with 0.1 ml 10 per cent oxazolone in acetone. Ear thickness was measured before ear painting and 24, 48, 72 and 96 hours afterwards.

Experiment B.7.

Forty-four male CFW mice were skin sensitized. Ten days later they were divided into 4 groups and treated as follows:-

Group 1 - 12 mice. 0.1 ml acetone intragastrically.

Group 2 - 12 mice. 0.1 ml 2% oxazolone/acetone intragastrically.

Group 3 - 10 mice. 0.1 ml 2% oxazolone/olive oil intragastrically.

Group 4 - 10 mice. 0.1 ml olive oil intragastrically.

Six hours after dosing, the animals were ear-tested, and the increase in ear/

ear thickness determined 24 hours later.

Experiment B.8.

The oxazolone/acetone component of Experiment B.7 was repeated for confirmation of the result. Thirty-four male CFW mice were skin sensitized, divided into 3 groups and treated as follows:-

Group 1 - 10 mice. No oral treatment.

Group 2 - 12 mice. 0.1 ml acetone intragastrically.

Group 3 - 12 mice. 0.1 ml 2% oxazolone/acetone intragastrically.

Six hours after dosing, the animals were ear-tested, and the increase in ear thickness determined 24 hours later. The mice were ear-tested again 3 weeks later.

Experiment B.9.

Forty-five Ash CS1 female mice were skin sensitized, divided into 4 groups and treated orally as follows:-

Group 1 - 10 mice. No treatment.

Group 2 - 11 mice. 0.1 ml olive oil for 4 days.

Group 3 - 12 mice. 0.1 ml 0.1% oxazolone/olive oil for 4 days.

Group 4 - 12 mice. 0.1 ml 1.0% oxazolone/olive oil for 4 days.

Four hours after the last oral dose, the animals were ear-tested and the increase in ear thickness determined 24 hours later.

GROUP C:- Mucosal challenge of skin sensitized animals.

Experiment C.1.

Ten guinea-pigs were skin sensitized to DNCB. Two weeks later, the animals were challenged by rectal instillation of 1 ml DNCB in an inert/

inert cellulose Pectin paste ('Orabase', E.R. Squibb) introduced by means of a 2 mm polyethylene catheter attached to a tuberculin syringe. The dose regime used was as follows:-

- 2 normal guinea-pigs - No treatment.
- 3 normal guinea-pigs - 1 g 0.25% DNCB/Orabase.
- 2 sensitized guinea-pigs - 1 g Orabase.
- 3 sensitized guinea-pigs - 1 g 0.1% DNCB/Orabase.
- 4 sensitized guinea-pigs - 1 g 0.25% DNCB/Orabase.

Twenty-four hours after challenge the animals were killed and the terminal colons removed, fixed in formal-saline and stained Haematoxylin and Eosin.

Experiment C.2.

Groups comprised of 2 sensitized female guinea-pigs were given single rectal instillations of 1.5 ml 0.25 per cent DNCB in olive oil for 2, 3 and 4 days. Three non-sensitized controls were treated for 4 days. Animals were killed 24 hours after challenge and the colon removed for histology.

Experiment C.3.

Groups of 5 skin sensitized guinea-pigs were given the following treatment:-

- Group 1 - 1.5 mg DNCB in olive oil emulsion intragastrically.
- Group 2 - 1.5 ml olive oil emulsion intragastrically.
- Group 3 - No treatment.

Three groups of 5 non-sensitized animals were similarly treated. Twenty-four hours after treatment, a piece of intestine 3 cm long was removed from the middle of the small intestine and processed for histology.

Experiment C.4.

Ten male and 10 female mice were skin sensitized and 10 days later given a single oral dose of 0.2 ml 1 per cent oxazolone in olive oil. Five male and 5 females were killed 24 and 48 hours after dosing, and the first 2 cm of intestine removed for histology. Ten male and 10 female non-sensitized mice were similarly treated.

Experiment C.5.

Thirty sensitized and 30 control female mice were given 0.1 ml 1 per cent oxazolone/olive oil orally for 1 to 6 days. Groups of 5 sensitized and 5 control mice were killed 24 hours after 1, 2, 3, 4, 5 and 6 daily doses and the first part of the small intestine processed for histology.

Experiment C.6.

Ten skin sensitized male and 5 female mice were given 0.2 ml 1 per cent oxazolone in olive oil orally at 3 hourly intervals over a period of 6 hours. Ten control male and 5 females were similarly treated. The mice were killed 24 hours later and the proximal small intestine processed for histology.

Experiment C.7.

Groups of 10 female skin sensitized mice were treated as follows:-

Group 1 - Undosed.

Group 2 - 0.2 ml 0.1% oxazolone in olive oil intragastrically.

Group 3 - 0.2 ml 1.0% oxazolone in olive oil intragastrically.

Group 4 - 0.2 ml 5.0% oxazolone in olive oil intragastrically.

Groups/

Groups of 10 non-sensitized mice were similarly treated. The first 2 cm of small intestine was removed 24 hours after dosing and processed for histology.

Experiment C.8.

Five groups of 5 skin sensitized female mice were treated orally as follows:-

Group 1 - Undosed.

Group 2 - 0.1 ml acetone-water (1:1).

Group 3 - 0.1 ml absolute alcohol.

Group 4 - 0.1 ml 2% oxazolone in acetone-water.

Group 5 - 0.1 ml 2% oxazolone in alcohol.

Groups of 5 control mice were similarly treated. The proximal small intestine was removed 24 hours later and processed for histology.

Experiment C.9.

Groups of 5 skin sensitized CFW mice were dosed per rectum as follows:

Group 1 - One dose 0.1 ml 1.0% oxazolone /olive oil.

Group 2 - One dose 0.1 ml 1.0% oxazolone/olive oil repeated 24 hours later.

Group 3 - One dose 0.1 ml 2.0% oxazolone in silicone grease.

Group 4 - One dose 0.1 ml 2.0% oxazolone in silicone grease repeated 24 hours later.

Groups of 5 normal mice were similarly treated. The mice were killed 24 hours after dosing and the terminal colon removed for histology.

GROUP D:- Oral administration followed by oral challenge.

Experiment D.1.

The survivors from Group B Experiment B.4 were used. These animals had been given oxazolone in olive oil orally in varying doses 3 times in one day and then skin sensitized. The mice were mixed and then divided into 3 groups of 10 mice for the following treatment:-

Group 1 - Undosed.

Group 2 - 0.2 ml olive oil intragastrically.

Group 3 - 0.2 ml 1% oxazolone in olive oil intragastrically.

The mice were killed 24 hours after challenge and the proximal small intestine removed for histology.

Experiment D.2.

The survivors from Group B Experiment B.1 were used. These mice had been given oxazolone in olive oil orally in varying doses once daily for 5 days and then skin sensitized. Five groups of 10 mice were given further oral administration as follows:-

Group 1 - Undosed.

Group 2 - 0.1 ml acetone.

Group 3 - 0.1 ml alcohol.

Group 4 - 0.1 ml 1.0% oxazolone in acetone.

Group 5 - 0.1 ml 1.0% oxazolone in alcohol.

The first 2 cm of small intestine was removed 24 hours after dosing.

Experiment D.3.

Twenty-five mice were given 0.1 ml 10 per cent oxazolone in acetone and then 10 days later divided into 5 groups of 5 mice and dosed orally/

orally as follows:-

Group 1 - Undosed.

Group 2 - 0.1 ml olive oil.

Group 3 - 0.1 ml acetone-water (1:1).

Group 4 - 0.1 ml 2% oxazolone in olive oil.

Group 5 - 0.1 ml 2% oxazolone in acetone-water.

Twenty-five normal mice were similarly treated. Twenty-four hours after challenge the proximal small intestine was removed for histology.

GROUP E:- Response of lymphoid tissues to oral sensitizers.

Experiment E.1.

Twenty-five guinea-pigs were given a single oral dose of 1.5 mg DNCB in olive oil emulsion. Groups of 5 animals were killed for histology on days 1, 2, 3, 4 and 6 after dosing. Sections were stained with Methyl green-pyronin.

Experiment E.2.

Groups of 5 guinea-pigs were given a single daily oral dose of 1.5 mg DNCB in olive oil emulsion for 2, 3 and 4 days. The animals were killed 24 hours after the last dose, and the mesenteric lymph node and spleen processed and stained with Methyl green-pyronin.

Experiment E.3.

Groups of 5 mice were given 0.1 ml 1 per cent oxazolone in olive oil orally for 1, 2, 3, 4, 5 and 6 days. The mice in each dose group were killed for histology 24 hours after their last dose.

Experiment E.4.

Mice/

Mice were given 0.1 ml 10 per cent oxazolone in acetone-water (2:1) orally and 5 were killed from 1 day to 6 days later. Control groups were given acetone-water only and a group of 5 undosed mice were also killed on day 1 in addition to the acetone-water control.

RESULTS.

GROUP A:- Skin sensitization.

Experiment A.1.

Painting one ear and one front foot with 0.02 ml 10 per cent oxazolone in absolute alcohol failed to produce skin sensitivity as assessed by ear challenge 10 days later (Table 8.1).

Experiment A.2.

Painting the shaved abdomen with 0.1 ml 10 per cent oxazolone for 3 consecutive days resulted in a marked response to ear challenge (Table 8.1).

Experiment A.3.

Acetone and olive oil were just as suitable as absolute alcohol as solvents for oxazolone when sensitization was attempted by painting the shaved abdomen (Table 8.1).

From these results, the standard skin sensitization regime adopted for subsequent experiments consisted of painting the shaved abdomen for 3 days with 10 per cent oxazolone in either acetone or alcohol.

When the above results were taken in conjunction with the results obtained in other experiments in this series, then it became clear that/

TABLE 8.1.

Response to ear challenge of mice sensitized
to oxazolone by different regimes.

Experiment number	Regime	Ear thickness before and 24 hr. after challenge. Mean \pm S.D.*			
		Sensitized		Controls	
		0 hr.	24 hr.	0 hr.	24 hr.
A.1	Ear and foot pad given single paint- ing with 0.02 ml 10% oxazolone in absolute alcohol.	180 \pm 21	185 \pm 29	175 \pm 19	180 \pm 15
A.2	Shaved abdomen painted with 0.1 ml 10% oxazolone in alcohol for 3 days.	210 \pm 29	430 \pm 62	195 \pm 16	210 \pm 20
A.3	Shaved abdomen painted with 0.1 ml 10% oxazolone in alcohol for 3 days.	165 \pm 14	350 \pm 47	185 \pm 16	190 \pm 27
	Shaved abdomen painted with 0.1 ml 10% oxazolone in acetone for 3 days.	200 \pm 18	410 \pm 17		
	Shaved abdomen painted with 0.1 ml 10% oxazolone in olive oil for 3 days.	185 \pm 14	395 \pm 21		

*Ear thickness given in arbitrary units. Each unit is
equivalent to 10^{-4} cm.

145 14

Last dose ... (10.1) ... ear test ... (?) ... ear pain ...
... (48-96 hr) ... skin pain ... (24-96 hr) ... measure
thickness.

Can't get the scheme

that the ear thickness of challenged unsensitized Ash CS1 mice never exceeded 250 units (the usual range being 150 - 225 units), and that challenge of skin sensitized mice resulted in ear thickness' greater than 300 units (usually 325 +). The ear measurement of 275 units was therefore taken as the borderline between a positive and negative response to challenge, 275 units and greater being regarded as a positive and 250 units or less as negative.

GROUP B:- Skin responsiveness following oral administration.

Experiment B.1.

Eight of the 20 guinea-pigs given 1.5 mg DNCB orally for 5 days became ill or died before skin testing and were discarded. Five of these animals died as a result of a ruptured oesophagus. Of the rest, one died and two became ill, but no lesions were seen on post mortem.

Four of the remaining 12 healthy guinea-pigs showed strong positive reactions to the skin tests, the intracutaneous test sites exhibiting erythematous lesions greater than 9 x 9 mm 24 hours after challenge.

Experiment B.2.

When mice were given various concentrations of oxazolone orally for 5 days only one mouse showed a positive response (275 +) when ear challenged 10 days later. When the mice were subsequently skin sensitized, and then ear tested for a second time, several of the mice still failed to show positive ear reactions, whereas mice not pretreated orally were all positive on challenge (Table 8.2).

Experiment B.3.

Experiment B.3 confirmed the results in Experiment B.2 where pretreatment with oxazolone in olive oil orally produced a depression in subsequent skin reactivity (Table 8.2). The 10 per cent dose level group was not repeated because of the high mortality rate found in Experiment B.2.

TABLE 8.2.

Ear responses of mice given oxazolone orally for 5 days and then the response to ear challenge after subsequent skin sensitization.

Regime	Number of positive ear responses on first ear test		Number of positive ear responses after skin sensitization	
	Experiment B.2	Experiment B.3	Experiment B.2	Experiment B.3
5 oral doses of 0.1 ml 0.01% oxazolone/olive oil.	0/14	0/15	13/14	14/15
5 oral doses of 0.1 ml 0.1% oxazolone/olive oil.	0/14	0/14	6/14	8/14
5 oral doses of 0.1 ml 1.0% oxazolone/olive oil.	0/15	0/15	12/15	9/15
3 oral doses of 0.1 ml 10% oxazolone/olive oil.	1/8	Not done	7/8	Not done
Untreated	0/10	0/5	10/10	5/5

Experiment B.4.

Mice given various concentrations of oxazolone in olive oil on 3 occasions on the same day failed to show positive reactions when ear-tested 10 days later. As in Experiments B.2 and B.3, subsequent skin painting failed to produce skin sensitivity in several mice as measured by the response to a second ear test (Table 8.3).

TABLE 8.3.

Ear response to mice given oxazolone orally
3 times in one day and subsequent response
after skin sensitization.

Regime	Number of positive ear responses on first ear test	Number of positive ear responses after skin sensitization
3 doses of 0.01% oxazolone in olive oil 3 hours apart.	0/11	10/11
3 doses of 0.1% oxazolone in olive oil 3 hours apart.	0/15	11/15
3 doses of 1.0% oxazolone in olive oil 3 hours apart.	0/14	6/14
3 doses of 10% oxazolone in olive oil 3 hours apart.	0/10	6/10
Untreated	0/7	7/7

Experiment B.5.

Mice dosed orally with various concentrations of oxazolone in either acetone or alcohol failed, with the exception of one mouse, to become/

become skin sensitized. When subsequent skin sensitization was attempted, several mice failed to show positive ear responses, particularly mice in the highest dose group (10 per cent oxazolone) (Table 8.4).

TABLE 8.4.

Response to ear challenge after oral dosing with oxazolone in either acetone or alcohol and then ear response after subsequent skin sensitization.

Regime	Number of positive ear responses on first ear test	Number of positive ear responses after skin sensitization
2 doses of 0.1 ml 0.1% oxazolone in alcohol 48 hours apart.	0/6	4/6
2 doses of 0.1 ml 1.0% oxazolone in alcohol 48 hours apart.	0/11	8/11
1 dose of 0.1 ml 10% oxazolone in alcohol.	1/10	3/10
1 dose of 0.1 ml 0.1% oxazolone in acetone - $\frac{1}{2}$ dose 48 hours later.	0/10	10/10
1 dose of 0.1 ml 1.0% oxazolone in acetone - $\frac{1}{2}$ dose 48 hours later.	0/10	9/10
1 dose of 0.1 ml 10% oxazolone in acetone.	0/12	6/12
Untreated	0/5	5/5

Experiment B.6.

In the preceeding experiments it was noted that some of the orally pretreated mice began to develop pink ears during the 3 days of skin painting following the first ear test. It was suspected that orally treated mice may have become primed to respond to oxazolone as a result of treatment, so that they developed an ear response to oxazolone remaining in the ear from the first ear test, during the early stages of the skin sensitizing regime. However, the results obtained in this experiment show that the early response seen on the ears during skin sensitization also occurred in animals not pretreated orally (Table 8.5).

TABLE 8.5.

Ear responses of animals skin painted with 10% oxazolone 24, 48 and 96 hours after ear testing.

Regime	Number of positive ear tests	Number of positive ear tests on the first 3 days of skin painting		
		Day 1	Day 2	Day 3
0.1 ml 0.1% oxazolone in acetone orally. 0.05 ml 48 hours later.	0/14	0/14	0/14	2/14
0.1 ml 1.0% oxazolone in acetone orally. 0.05 ml 48 hours later.	0/14	0/14	0/14	6/14
0.1 ml acetone orally. 0.05 ml 48 hours later.	0/14	0/14	0/14	4/14

Experiment B.7.

Oral administration of 2 per cent oxazolone in acetone to sensitized mice partially inhibited the increase in ear thickness when the mice were tested 6 hours later. Oxazolone in olive oil had no effect (Table 8.6).

TABLE 8.6.

Effect of oral pretreatment with oxazolone on the subsequent ear response of sensitized mice.

Time interval	0.1 ml acetone	0.1 ml 2% oxazolone/acetone	0.1 ml 2% oxazolone/olive oil	0.1 ml olive oil
-6 hr.	267 \pm 25	260 \pm 20	275 \pm 20	245 \pm 20
0 hr.	271 \pm 23	256 \pm 16	270 \pm 23	248 \pm 18
24 hr.	421 \pm 63	343 \pm 60	433 \pm 74	428 \pm 43
% increase	55	34	60	73

Experiment B.8.

Experiment B.8 confirmed the results of Experiment B.7 in that oral administration of oxazolone in acetone to skin sensitized mice depresses the response to ear challenge. Acetone alone also depressed the response, but not to the same degree as oxazolone/acetone. This depression was no longer evident when the animals were retested 3 weeks later (Table 8.7).

TABLE 8.7.

Effect of oral pretreatment with oxazolone in acetone 6 hours before ear testing and on the response to a second ear challenge 3 weeks later.

	Time interval	Untreated	Acetone	2% oxazolone/acetone
Challenge I	-6 hr.	263 \pm 21	263 \pm 20	256 \pm 15
	0 hr.	265 \pm 13	268 \pm 20	260 \pm 19
	24 hr.	440 \pm 42	389 \pm 56	288 \pm 47
	% increase	66	45	11
Challenge II	0 hr.	260 \pm 13	257 \pm 16	255 \pm 19
	24 hr.	468 \pm 26	439 \pm 42	450 \pm 57
	% increase	80	71	76

Experiment B.9.

Oral dosing of skin sensitized mice with 0.1 per cent and 1.0 per cent oxazolone in olive oil for 4 days had no effect on the response of these mice to ear challenge (Table 8.8). Thus, repeated dosing with oxazolone/olive oil failed to reproduce the depression obtained using a single dose of oxazolone/acetone.

TABLE 8.8.

Effect of oral treatment with oxazolone in olive oil for 4 days on the subsequent response to ear challenge.

Time	Ear thickness before and after challenge in mice given the following pretreatments:-			
	Untreated	Olive oil	0.1% oxazolone	1.0% oxazolone
0 hr.	188 \pm 30	189 \pm 17	185 \pm 17	198 \pm 13
24 hr.	335 \pm 50	361 \pm 60	371 \pm 54	394 \pm 47
% increase	78	91	101	99

GROUP C:- Mucosal challenge of skin sensitized animals.

Experiment C.1.

Rectal instillation of 0.25 per cent DNCB in orabase into skin sensitized guinea-pigs elicited a reaction in 2 out of the 4 animals challenged. The reaction consisted of prominent oedema and congestion of the lamina propria which together with the marked mononuclear cell infiltration resulted in a distinct separation of adjacent crypt walls from each other, as well as separation of the crypts from the muscle layers. The mucosa of the crypts was intact and showed only slight lymphocytic infiltration. The venules and small veins in the muscle layers exhibited conspicuous perivascular cuffing with mononuclear cells (Plates 8.2, 8.3 and 8.4).

In normal guinea-pigs challenged with 0.25 per cent DNCB in orabase and in sensitized guinea-pigs challenged with 0.1 per cent DNCB there was only slight oedema of the submucosa of the colon and sparse cellular infiltration (Plate 8.1).

Experiment C.2.

Sensitized guinea-pigs given rectal instillation of 1.5 ml 0.25 per cent DNCB in olive oil for 2, 3 or 4 days had lesions in the colon similar to those described in Experiment C.1 above, namely marked oedema and cellular infiltration. Only slight oedema and moderate cellular infiltration were seen in the 3 normal guinea-pigs dosed for 4 days.

Experiment C.3.

When skin sensitized guinea-pigs were given 1.5 ml olive oil emulsion/

Plate 8.1 Colon of normal guinea-pigs 24 hours after rectal instillation of 0.25% DNCB/Orabase. Note very slight oedema. Haematoxylin and eosin x 200 (approx.).

Plate 8.2 Colon of a sensitized guinea-pig 24 hours after rectal instillation of 0.25% DNCB/Orabase. Note the accumulation of mononuclear cells at the base of the crypts. Haematoxylin and eosin x 200 (approx.).

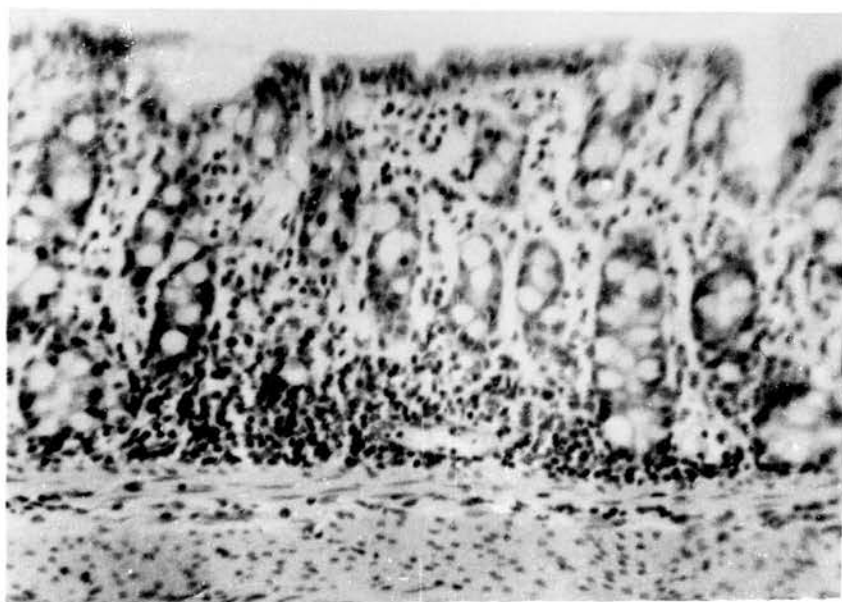
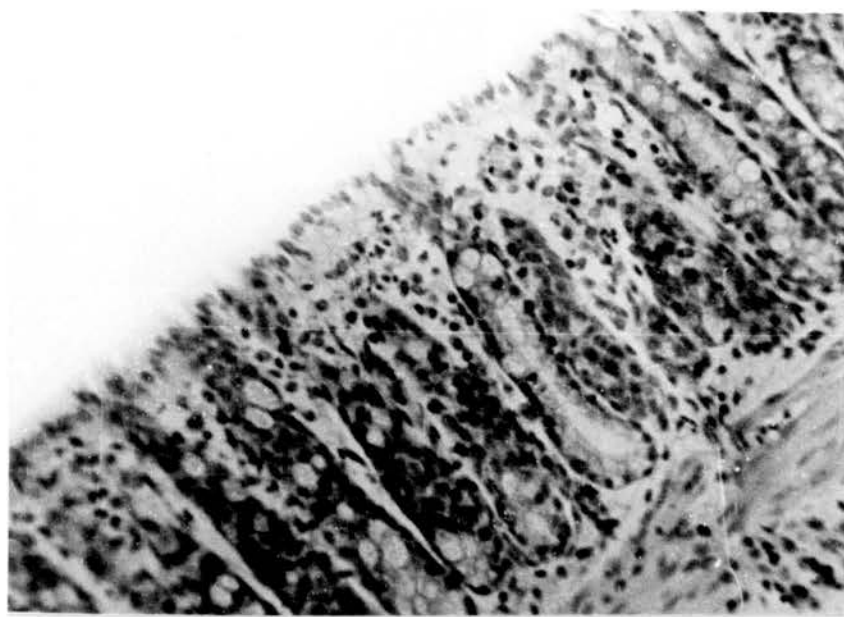
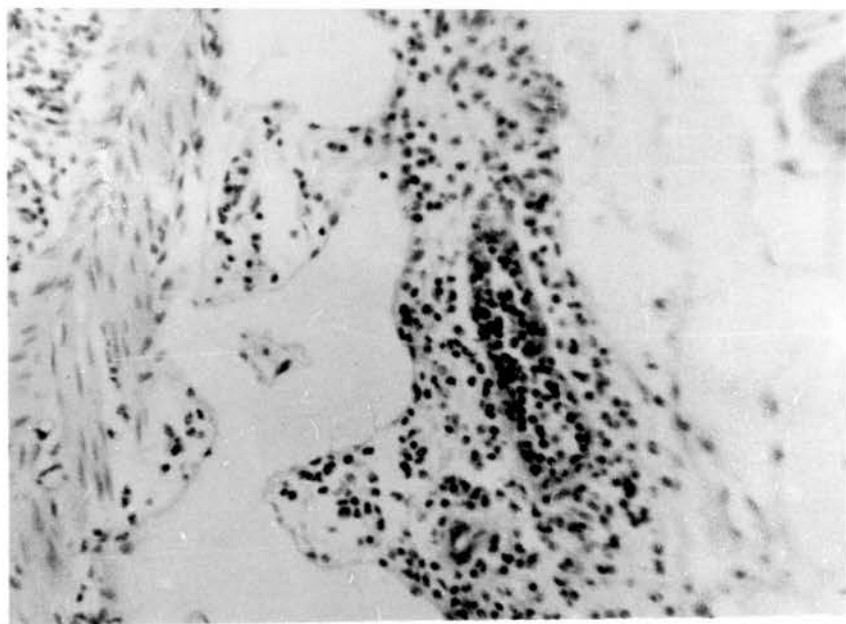
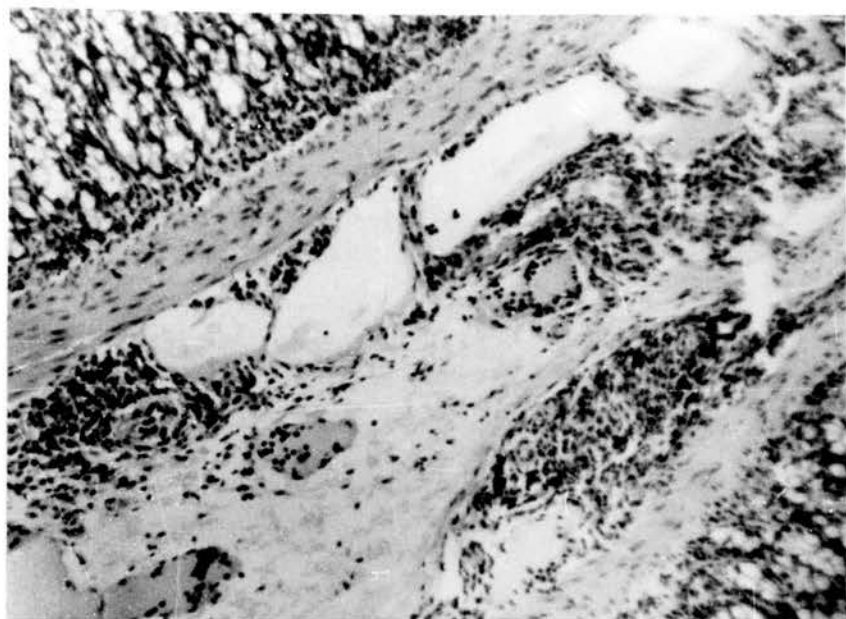


Plate 8.3 Colon of a sensitized guinea-pig
24 hours after the rectal
instillation of 0.25% DNCB/Orabase.
Note the marked cellular response
in the adventitia.
Haematoxylin and eosin x 60
(approx.).

Plate 8.4 Perivascular cuffing reaction in
a lesion produced in the colon of
sensitized guinea-pigs by rectal
instillation of 0.25% DNCB/Orabase.
Haematoxylin and eosin x 125
(approx.).



emulsion containing 1.5 mg DNCB, no gross lesions were visible in the intestine on post-mortem examination 24 hours later. When the number of lymphocytes per 1000 epithelial cells was determined for a piece of intestine taken from approximately the centre of the small intestine, the results showed that there was no significant increase in the number of lymphocytes when compared to the controls (Table 8.9).

TABLE 8.9.

Number of lymphocytes per 1000 epithelial cells in a section of small intestine from normal and sensitized guinea-pigs 24 hours after a single dose of 1.5 mg DNCB.

Regime	Number of lymphocytes per 1000 epithelial cells. Mean \pm S.D.	Regime	Number of lymphocytes per 1000 epithelial cells. Mean \pm S.D.
Normal guinea-pig undosed.	356 \pm 130	Sensitized guinea-pig undosed.	493 \pm 142
Normal guinea-pig olive oil.	408 \pm 100	Sensitized guinea-pig olive oil.	527 \pm 88
Normal guinea-pig DNCB (1.5 mg).	476 \pm 146	Sensitized guinea-pig DNCB (1.5 mg).	650 \pm 102

Experiment C.4.

Oral administration of 0.2 ml 1 per cent oxazolone in olive oil to skin sensitized mice had no significant effect on the numbers of lymphocytes in the epithelium of the proximal small intestine when the animals were killed 24 or 48 hours later (Table 8.10).

TABLE 8.10.

Number of lymphocytes in the villus epithelium 24 and 48 hours after oral dosing of control and skin sensitized mice with 0.2 ml 1% oxazolone in olive oil.

Group	Sex	Lymphocytes/10 V.U. ± S.D.	
		24 hr.	48 hr.
Sensitized	M	427 ± 84	496 ± 76
	F	378 ± 45	467 ± 58
Control	M	387 ± 87	452 ± 90
	F	412 ± 67	366 ± 81

Experiment C.5.

Daily administration of 0.1 ml 1.0 per cent oxazolone in olive oil to skin sensitized mice had no effect on the number of lymphocytes in the intestinal epithelium even though administration was continued for up to 6 days (Table 8.11).

TABLE 8.11.

Effect of repeated oral dosing with 0.1 ml 1% oxazolone on the number of lymphocytes in the intestinal epithelium.

Group	Lymphocytes/5 V.U. ± S.D.					
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Sensitized	137 ± 49	118 ± 55	128 ± 32	180 ± 49	157 ± 30	143 ± 23
Control	114 ± 51	184 ± 40	140 ± 46	105 ± 37	136 ± 50	164 ± 26

Experiment C.6.

Oral dosing of skin sensitized mice with 1 per cent oxazolone in olive oil on 3 occasions during one day had no effect on the numbers of lymphocytes in the intestinal epithelium (Table 8.12).

TABLE 8.12.

The effect of 3 oral doses of 1% oxazolone, given on the same day, on the number of lymphocytes in the intestinal epithelium.

Group	Sex	Lymphocytes/10 V.U. ± S.D.
Sensitized	M	340 ± 90
	F	339 ± 90
Control	M	375 ± 94
	F	351 ± 67

Experiment C.7.

Oral administration of different concentrations of oxazolone in olive oil failed to increase the numbers of lymphocytes in the intestinal epithelium. In fact, within 24 hours after dosing, the numbers of lymphocytes was decreased in the highest dose group (5 per cent oxazolone) (Table 8.13).

Although there was little reaction in the intestine, the stomachs of the mice, both control and sensitized, receiving the 5 per cent dose were congested and oedematous. It was not possible to distinguish between the lesions of the control and sensitized mice macroscopically, but histologically, the keratinized region of the stomachs from sensitized mice/

mice showed very marked cell infiltration in addition to the oedema which was found in both control and sensitized mice. The connective tissue beneath the mucosa of the glandular portion of the stomach was also oedematous, but cell infiltration was minimal and there was no obvious effect on the actual mucosa itself.

TABLE 8.13.

The number of lymphocytes in the villus epithelium of control and skin sensitized mice after oral administration of different concentrations of oxazolone in olive oil.

Group	Lymphocytes/5 V.U. \pm S.D.			
	Undosed	0.1%	1.0%	5.0%
Sensitized	204 \pm 47	228 \pm 38	193 \pm 65	162 \pm 55
Control	194 \pm 45	194 \pm 33	202 \pm 44	151 \pm 22

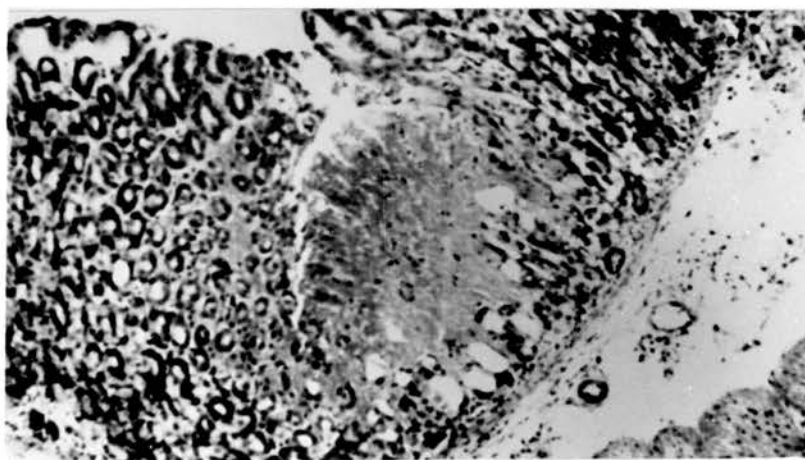
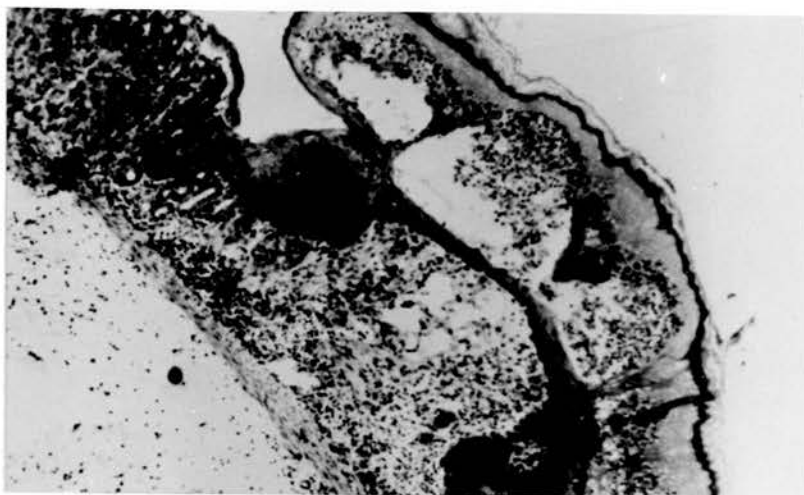
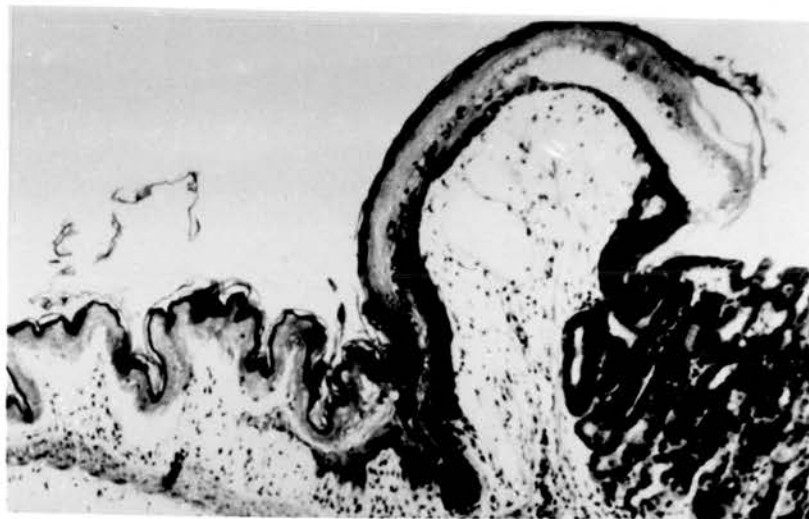
Experiment C.8.

Oral administration of 2 per cent oxazolone in either acetone or alcohol had no effect on the number of lymphocytes in the intestinal epithelium. However, lesions were seen both macroscopically and histologically in the stomach. Congestion of the pyloric region of the stomach was observed in both sensitized or control mice receiving either acetone or alcohol only. Mice receiving oxazolone in either alcohol or acetone often had large (up to 5 mm diameter) fluid-filled vesicles in the cardiac portion of the stomach, in addition to marked congestion of the pyloric region. It was not possible to distinguish between sensitized and/

Plate 8.5 The effect of oral administration of 2% oxazolone in either alcohol or acetone on the keratinized region of the normal mouse stomach. Note vesiculation of the epithelium and oedema of the connective tissue. Haematoxylin and eosin x 75 (approx.).

Plate 8.6 The effect of oral administration of 2% oxazolone in either alcohol or acetone on the keratinized region of the sensitized mouse stomach. Note the very marked cell infiltration compared with Plate 8.5. Haematoxylin and eosin x 75 (approx.).

Plate 8.7 Focal coagulative necrosis of the glandular portion of the mouse stomach caused by oral dosing with ethanol. Haematoxylin and eosin x 100 (approx.).



and control lesions by macroscopic assessment.

Histologically, it was found that administration of alcohol produced focal coagulative necrosis in the pyloric mucosa (Plate 8.7). Vesiculation and oedema was observed in the cardiac region of animals receiving oxazolone in either acetone or alcohol, but as in Experiment C.7 marked cellular infiltration was characteristic only of sensitized mice and not of control mice (Plates 8.5 and 8.6).

TABLE 8.14.

Effect of oral administration of 2% oxazolone in different vehicles on the number of lymphocytes in the intestinal epithelium.

Group	Lymphocytes/5 V.U. \pm S.D.				
	Undosed	Acetone	Alcohol	Oxazolone in acetone	Oxazolone in alcohol
Control	226 \pm 66	200 \pm 34	242 \pm 55	213 \pm 42	175 \pm 27
Sensitized	189 \pm 26	177 \pm 30	195 \pm 18	200 \pm 48	165 \pm 51

Experiment C.9.

Administration of oxazolone in either olive oil or silicone grease for one or two days failed to produce a hypersensitivity reaction in the colon similar to that found in the guinea-pig using DNCB.

GROUP D:- Oral administration followed by oral challenge.

Experiment D.1.

Oral administration of 1 per cent oxazolone in olive oil to mice that had been previously exposed to oxazolone orally and then skin sensitized/

sensitized failed to influence the number of lymphocytes in the intestinal epithelium (Table 8.15).

TABLE 8.15.

Oral challenge of orally pretreated mice.

Regime	Lymphocytes/5 V.U. ± S.D.
Undosed	232 ± 22
0.2 ml olive oil	236 ± 31
0.2 ml 1% oxazolone	241 ± 34

Experiment D.2.

Oral administration of 1.0 per cent oxazolone in either acetone or alcohol to mice that had been previously exposed to oral oxazolone and then skin sensitized had no effect on the number of lymphocytes in the proximal small intestinal epithelium (Table 8.16).

TABLE 8.16.

Oral challenge of orally pretreated mice.

Regime	Lymphocytes/5 V.U. ± S.D.
Undosed	292 ± 43
0.1 ml acetone	296 ± 61
0.1 ml alcohol	271 ± 22
0.1 ml 1.0% oxazolone/acetone	301 ± 47
0.1 ml 1.0% oxazolone/alcohol	296 ± 36

Experiment D.3.

A second oral administration of oxazolone to mice given 10 per cent oxazolone 10 days earlier failed to increase the number of lymphocytes in the small intestinal epithelium (Table 8.17).

TABLE 8.17.

The effect of a second oral dose of oxazolone on the number of lymphocytes in the small intestinal epithelium of mice given a primary exposure 10 days earlier.

Group	Lymphocytes/5 V.U. \pm S.D.				
	Undosed	Olive oil	Acetone	2% oxazolone/ olive oil	2% oxazolone acetone
Sensitized	205 \pm 58	218 \pm 83	133 \pm 46	190 \pm 47	130 \pm 26
Control	114 \pm 38	116 \pm 38	136 \pm 64	138 \pm 48	149 \pm 34

Histological examination of the stomach revealed the presence of chronic ulcers in the cardiac region of animals treated with oxazolone 10 days earlier. It was therefore, impossible to detect any response in the keratinized epithelium to challenge because of this lesion. However, the glandular part of the stomach was relatively unaffected by previous exposure and there was no response in the region to challenge.

GROUP E:- Response of the lymphoid tissues to oral sensitizers.

Experiment E.1.

Sections from a central portion of mesenteric lymph node and spleen were examined from groups of 5 guinea-pigs killed 1, 2, 3, 4 and

TABLE 8.18.

Changes in the cellularity of the splenic red pulp following a single oral dose of DNCB.

Time after DNCB	Guinea-pig number				
	1	2	3	4	5
24 hr.	+	+	0	++	0
48 hr.	+	0	0	0	0
72 hr.	+++	+++	0	+++	++
96 hr.	++	0	0	0	+
144 hr.	0	++	++	0	+

6 days after oral administration of 1.5 mg DNCB in olive oil emulsion. Sections from normal animals were also examined. After staining with Methyl green-pyronin, it was possible to identify 4 conspicuous features in the mesenteric lymph node, medulla, germinal centres, large aggregates of deeply staining lymphoid cells in the cortex and diffuse pale staining cortical areas which merged with the medulla. On the basis of the size and number of these zones it was not possible to differentiate between normal lymph nodes and nodes from animals given DNCB, nor was any increase in the number of large pyroninophilic cells observed in the cortex.

There were no consistent changes in the white pulp of the spleen in terms of the size of the periarteriolar follicles, or in the incidence of germinal centres, and it was not possible to differentiate slides from different time intervals on the basis of these parameters. The red pulp showed the most marked changes in cellularity. Three out of 5 spleens taken from guinea-pigs killed 72 hours after DNCB showed a marked/

marked red pulp hyperplasia, consisting mainly of large pyroninophilic cells and cells of the plasmacytic series. Adjacent to the red pulp sinusoids, and often in the lumen, small cells with deeply stained nuclei predominated. Many, but not all, of these cells had a thin rim of pyroninophilic cytoplasm. The red pulp hyperplasia was graded as 0 to +++ and the results for the different time intervals is presented in Table 8.18.

Experiment E.2.

It was not possible to differentiate between mesenteric lymph nodes taken from guinea-pigs given 1.5 mg DNCB orally for 2, 3 or 4 days in terms of the size and cell content of the medulla, germinal centres and diffuse cortical zones.

In contrast to Experiment E.1 there was no significant change in the cellularity of spleens taken from guinea-pigs given repeated doses of DNCB (Table 8.19).

TABLE 8.19.

Changes in the cellularity of the splenic red pulp after dosing with DNCB at 24 hourly intervals. Guinea-pigs killed 24 hours after last oral dose.

Number of doses of DNCB	Guinea-pig number			
	1	2	3	4
2	0	+	0	+++
3	+	+	+	+
4	+	0	+	+

Experiment E.3.

It was not possible to differentiate between mesenteric lymph nodes taken from mice given 0.1 ml 1 per cent oxazolone in olive oil orally for 1, 2, 3, 4, 5 or 6 days when sections were scanned under low power. When the number of large pyroninophilic cells in a high power field of 3 different paracortical areas of the node was determined, it was found that there was quite marked variation between nodes from different mice in the same time interval (Table 8.20), and even between different paracortical areas of the same node. Thus, it was not possible to differentiate between nodes from different time intervals on the basis of pyroninophil cell content. An equally marked variation was found when sections of spleens, from animals killed at the same time interval, were scanned for gross changes in the cellularity of the red and white pulp.

TABLE 8.20.

Mean pyroninophil cell count (of 3) in paracortical areas of mesenteric lymph nodes from mice killed after multiple dosing with 1% oxazolone in olive oil.

Number of doses of oxazolone	Mean pyroninophil cell count in mouse number					Mean \pm S.D.
	1	2	3	4	5	
1	16	18	23	14	11	16.4 \pm 4.5
2	13	13	32	29	27	22.8 \pm 9.2
3	20	19	46	28	15	25.6 \pm 12.4
4	16	22	16	10	18	16.4 \pm 4.4
5	9	18	14	19	8	13.6 \pm 5.0
6	14	14	22	15	16	16.2 \pm 3.4

Experiment E.4.

No differences could be seen on low power scanning of sections of mesenteric lymph nodes taken from mice at various times after oral dosing with 10 per cent oxazolone in acetone/water. With the exception of one node at 72 hours there was no difference in the number of large pyroninophilic cells in the paracortical areas of nodes (Table 8.21). The marked variation in spleen cellularity noted in Experiment E.3 were also observed in this experiment.

TABLE 8.21.

Mean pyroninophil cell count (of 3) in paracortical areas of mesenteric lymph nodes of mice killed at various time intervals after a single oral dose of 10% oxazolone.

Regime	Time after dosing (hr.)	Mean pyroninophil cell count in mouse number					Mean \pm S.D.
		1	2	3	4	5	
Single dose of 0.1 ml 10% oxazolone in acetone/water.	24	9	16	14	15	9	12.6 \pm 3.4
	48	8	22	5	8	6	9.8 \pm 6.9
	72	7	12	13	19	46	19.4 \pm 15
	96	6	9	11	12	16	10.8 \pm 3.7
	120	7	7	10	14	14	10.4 \pm 3.5
	144	8	11	11	13	16	11.8 \pm 2.9
Single dose of 0.1 ml acetone/water.	24	5	12	6	9	5	7.4 \pm 3.0
	48	8	11	12	13	14	11.6 \pm 2.3
	72	7	9	16	17	17	13.2 \pm 4.8
	96	5	8	11	13	22	11.8 \pm 6.5
	120	7	10	11	13	21	12.4 \pm 5.3
	144	6	6	10	10	15	9.4 \pm 3.7
Undosed	-	5	3	13	9	6	7.2 \pm 3.9

DISCUSSION.

The results obtained in this series of experiments failed to support conclusively the hypothesis that lymphocytes in the intestinal epithelium are involved in delayed-type hypersensitivity reactions. Attempts to sensitize animals by oral administration of sensitizers were generally unsuccessful, as were attempts to orally challenge skin sensitized animals, even though several variations of dose, vehicle and duration of administration were used. The tendency for orally administered sensitizing agents to produce a state of tolerance rather than hypersensitivity is in agreement with the results obtained by several other groups of workers (Chase, 1946; Battisto and Chase, 1955; Coe and Salvin, 1965; Pomeranz and Norman, 1966; Asherson and Ptak, 1970; Pomeranz, 1970). A few animals, particularly guinea-pigs, did become sensitized, although this was the exception rather than the rule, and may have been the result of accidental contamination of the lips during dosing, even though every attempt was made to avoid this.

Immunological tolerance in delayed hypersensitivity reactions has been a rather neglected phenomenon, but one or two hypotheses have been put forward to explain it, based largely on the more numerous studies of tolerance in relationship to antibody production. The original hypothesis outlined by Medawar (1960) suggests that tolerance is produced when antigen reacts directly with immunocompetent cells. These immunocompetent cells are probably thymus-derived lymphocytes in the circulating pool (Miller and Mitchell, 1970). Evidence in favour of this hypothesis is accumulating from in vitro and cell transfer studies. Lymphocytes from normal animals are known to combine directly with antigen/

antigen in vitro (Sulitzeanu, 1968; Byrt and Ada, 1969; Sulitzeanu and Naor, 1969), and when lymphoid tissue or lymphoid cells which have been exposed to antigen in vitro are injected into irradiated recipients, there is a marked reduction in the capacity of the recipient to respond to specific antigenic stimulation (Britton, 1969; Diener and Armstrong, 1969; Scott and Waksman, 1969). Thus, it would appear that with certain antigens, direct interaction of antigen with lymphocytes leads to a state of tolerance. The fate of these complexed lymphocytes and their exact relationship to the phenomenon of tolerance is unknown, but they may be destroyed or lost from the body since tolerant mice have fewer antigen-binding lymphocytes than normal mice (Naor and Sulitzeanu, 1969).

The type of tolerance found in delayed hypersensitivity reactions could also be explained on the basis of a direct interaction of antigen with lymphocytes. Evidence for this is found in the cell transfer studies of Scott and Waksman (1969), who found that recipients of bovine gamma globulin-treated lymphoid tissue had a reduced delayed reactivity. The evidence for simple chemical sensitization and tolerance is more circumstantial. The work of Macher and Chase (1969) suggests that it is the sensitizer retained in the skin, as protein-hapten complexes, which is responsible for sensitization, whereas, sensitizer escaping into the blood stream leads to tolerance (if the skin complexed site is removed). The importance of sensitizer escaping into the blood stream, and thus becoming capable of reacting directly with lymphocytes, is emphasized by the studies which demonstrate the production of an unresponsive or tolerant state by intravenous injection of simple chemicals/

chemicals (Battisto and Miller, 1962; Frey, Geleick and de Weck, 1964(a); Crowle and Hu, 1970). Attempts to demonstrate directly the importance of antigen-lymphocyte complexing have met with variable success. Battisto and Bloom (1966) produced tolerance to picryl chloride by a single intravenous injection of picrylated spleen cells, whereas, Baumgarten and Geczy (1970) actually induced sensitivity in guinea-pigs by the intraperitoneal injection of dinitrophenylated autologous lymphocytes.

2 The alternative hypothesis to lymphocyte-antigen interaction is that, under certain conditions, pretreatment of the animals with sensitizer leads to the production of anti-hapten antibody which combines with the hapten moiety of the skin complexes when subsequent skin sensitization is attempted, and thus, effectively prevents the induction of delayed hypersensitivity. However, this hypothesis is unlikely for several reasons. Tolerance persists for several months (Chase, 1946), whereas blocking antibody levels would be expected to decline over this period. More directly, Pomeranz (1970) found that feeding picryl chloride did not result in antibody production, as assessed by the PCA test, and moreover, subsequent attempts to induce antibody production failed, thus confirming the findings by Chase that induction of antibody production, as well as contact sensitivity, is depressed by oral administration of sensitizers (Chase, 1949; Battisto and Chase, 1955; 1965).

If the hypothesis that tolerance is caused by direct interaction of sensitizing chemicals with immunologically competent lymphocytes is accepted, then it is necessary to explain the observation that, whereas application/

application of these chemicals to the skin generally produces sensitization (see Lowney (1965), for exceptions), oral administration generally produces tolerance. If one accepts the statement by Crowle (1966) that response and tolerance are so interrelated that they can be considered mutually interacting antipodes of the same phenomenon, then the explanation could lie in the different types of epithelium involved. Thus, in an epithelium such as the skin, sufficient hapten complexes with skin proteins to ensure that sensitivity predominates over the tolerance produced by sensitizer escaping into the blood stream, whereas in the intestinal epithelium, the reverse is true, and direct interaction of sensitizer with lymphocytes predominates over epithelial complexing.

Thus, the type of epithelium may influence the actual dose level entering the body. However, the relationship of dose level to tolerance induction is somewhat obscure. High and low zone levels of tolerance have been demonstrated in antibody producing systems (Mitchison, 1964; 1968; Shellam and Nossal, 1968; Shellam, 1969), and Macher and Chase (1969) have suggested that the tolerance in contact hypersensitivity produced by feeding sensitizers is a low zone tolerance. Some of the results in this study suggest that low oral doses may be more effective than high doses in producing tolerance, but other workers have found that increasing the dose level increases the number of tolerant animals (Coe and Salvin, 1963; Pomeranz, 1970). The different results may be due to the different chemicals and the different species that have been used in these experiments, and it is not yet possible to conclude whether or not oral administration produces high or low zone tolerance, or even whether the states of high and low zone tolerance exist in contact sensitization/

sensitization reactions.

Not only was sensitization rare after oral administration of sensitizers, but there was also no response in the small intestinal epithelium when oral challenge of skin sensitized animals was attempted. It is possible that this failure to produce challenge responses may have been due to technical reasons, and in particular, the time that the sensitizer was in contact with the mucosa. Preliminary studies with dye-stained vehicles showed that the vehicle rapidly entered the small intestine, and within 30 minutes, a large proportion of the small intestine was coloured, so there is no doubt that the sensitizer actually enters the lumen of the intestine. However, it is not possible to state that the contact time with the mucosa is adequate. On the other hand, if the responses found in the stomach are taken into account, then it can be seen that a contact time sufficient to produce lesions in the cardiac region of the stomach, has no effect on the glandular portion, which presumably has the same time of contact. Therefore, it could be postulated that the mucous coat acts as a protective barrier, but since oral administration produces tolerance, and in some cases sensitization, then at least a small proportion of the sensitizer must cross the mucosal barrier. On these grounds, it is possible to justify the statement that sensitizing chemicals complexing with the intestinal mucosa of skin sensitized animals does not result in the manifestation of a contact hypersensitivity reaction.

There are two possible explanations of this failure of oral challenge, one of which is related to tolerance induction, and the other which postulates antigen specificity for the reaction. If it is assumed that/

that the distribution of an oral challenge dose is similar to that of an oral dose which produces tolerance, then this leads to the question of the fate of orally administered sensitizers. Although the final answer will lie in radiotracer studies, it is possible to put forward a hypothesis on the basis of some of the evidence available in the literature. In the above discussion of tolerance it was suggested that the amount of sensitizer entering the blood stream seemed to play a role in the induction of tolerance. Since intravenous injection and oral administration of sensitizers both produce tolerance, then it could be suggested that a significant amount of sensitizer enters the blood stream following oral administration. This suggestion is supported by the similarity in skin reactivity of sensitized animals which have been given sensitizers either intravenously or orally prior to skin testing. Intravenous administration of sensitizer depresses the response of sensitized guinea-pigs to skin challenge (Frey, Geleick and de Weck, 1964(b); Frey, de Weck and Geleick, 1964; de Weck, Frey and Geleick, 1964; Polak and Turk, 1968), and the results found in this study show that prior oral administration of oxazolone in acetone, but not olive oil, depresses the subsequent response to ear challenge in mice. From these results it could be concluded that the failure to produce local challenge responses in the small intestinal epithelium is due to the absorption of sensitizer and the formation of sensitizer complexes elsewhere in the body, or perhaps direct interaction with sensitized lymphocytes (Geczy and Baumgarten, 1969), which prevent the manifestation of a local response. However, the fact that positive responses are produced locally in the cardiac region of the stomach would tend to exclude the hypothesis/

hypothesis of a central inhibition of local epithelial reactions.

The alternative hypothesis to a central blocking of local reactions is that contact hypersensitivity reactions are epithelium specific. Studies with hapten-protein complexes indicate that the specificity of delayed-type reactions resides in the protein part of the complex rather than the hapten (Benacerraf and Gell, 1959; Salvin and Smith, 1960; Gell and Benacerraf, 1961; Salvin and Smith, 1961; 1964). On this basis it could be suggested that the small intestinal protein-hapten complexes formed after oral challenge do not act as eliciting antigens in skin sensitized animals. However, complexes formed in the keratinized epithelium of the cardiac region of the stomach are probably equivalent to those formed in the keratinized epithelium of the skin. Therefore, this hypothesis would explain the production of lesions in the cardiac portion of the stomach, but not in the pyloric region or in the small intestine. Unfortunately, it is possible to produce contact reactions in the colon of the guinea-pig, so unless the colon, but not the small intestine, forms similar complexes to those found in the cardiac region of the stomach and in the skin, the hypothesis is not correct. Oral challenge of animals previously exposed to sensitizers by the oral route also failed to produce any reaction in the small intestine and therefore the hypothesis that sensitization and challenge have to be via the same type of epithelium must remain unproven.

Although the results in this series of experiments failed to support directly the hypothesis that lymphocytes in the intestinal epithelium are involved in delayed-type hypersensitivity reactions, the hypothesis may still be correct. Under the prevailing experimental conditions/

conditions, the small intestine would appear to be resistant to active induction of contact sensitivity, in fact the reverse is true, and tolerance is produced. If tolerance and sensitivity can be regarded as antipodes of the cellular immune response, then it may be that all the experimental work to date has involved what might be called the wrong end of the scale, whereas in the normal animal, the intestine is active in that part of the scale which is finely tuned to give the host maximum cellular protection without excessive self-injury in the form of a hypersensitive reaction. Perhaps an analogy might be drawn with certain asthmatic conditions in humans, which are allergic in origin. Most humans show no clinical reaction to antigen inhalation, whereas certain humans overreact. The delayed hypersensitivity equivalent of this pulmonary anaphylactic reaction may be manifested in Crohn's disease and ulcerative colitis, whereby the immune mechanisms in certain humans are 'out of tune' and they overact in terms of cellular hypersensitivity to antigens present in the intestinal lumen. As a final note on the analogy with the respiratory tract, it is interesting to note that anaphylactic reactions in the lung can be inhibited by repeated exposure to aerosols (Swineford, 1967; Hicks, Okpako and Leach, 1968; Bartlema and Brannins, 1969).

In summary, oral administration of relatively large amounts of skin sensitizing agents generally produces tolerance rather than sensitization. Similarly, the small intestine does not respond by means of a contact hypersensitivity reaction to oral administration of compounds to which the animal has been skin sensitized. Although the results may not support the hypothesis that lymphocytes in the intestinal epithelium are/

are participating in delayed hypersensitivity reactions, it is suggested that these cells are in fact doing so, but that under the experimental conditions employed, another aspect of cellular or delayed reactivity, that of tolerance, was being evoked.

CHAPTER 9.SUMMARY OF RESULTS AND DISCUSSION.

This discussion will continue the theme outlined in Chapter 1, namely, a summary of the properties of the population of lymphoid cells in the intestinal epithelium followed by an evaluation of the possible functions of this population in the light of these properties, and those outlined by other experimental investigations.

The incidence of lymphocytes in the intestinal epithelium of the mouse is extremely variable. Mice in this study, housed under conventional conditions, had a ratio of 4 epithelial cells to 1 lymphocyte, which, with the exception of the figures quoted by Andrew and Andrew (1945), is well above the figure of approximately 10:1 which is generally reported in the literature for the mouse and for other species. Although the incidence varied between different mice, it remained fairly constant throughout the length of the intestine of any one individual. The majority of the lymphocytes were close to the basement membrane and situated between the epithelial cells, in agreement with the results of Meader and Lander (1967). In terms of their nuclear and cytoplasmic morphology, then the lymphocytes in the intestinal epithelium must be regarded as a heterogeneous population. Nuclear diameter ranged from 3.0 μm - 8.0 μm in sections, and both leptochromatic and pachychromatic nuclei were observed. Electron microscope studies indicated a variation in cytoplasmic morphology from scanty monoribosomal-containing cytoplasm to abundant polyribosomal-containing cytoplasm. This was reflected in the varying degrees of pyroninophilia seen by light microscopy. Occasional plasma cells were observed by light, but not electron microscopy./

microscopy.

Studies of the cell labelling after the injection of tritiated thymidine have shown that at least two populations can be distinguished, one of which has a relatively short life span, and one which is relatively long-lived. The possibility of a third population with a medium life span was suggested by studies of the labelling index following repeated administration of tritiated thymidine. On the assumption that long-lived lymphocytes are cortisone-resistant (Schrek and Batra, 1966), then the failure of prolonged doses of betamethasone to completely deplete the intestinal lymphocyte population confirmed the heterogeneity in life span observed in the autoradiographic studies.

The origin and fate of the epithelial lymphocyte population is still not completely clear. Since some of the cells in the epithelium divide, then the population must be in part self-replicating. However, light and electron microscopy have shown that cells cross the basement membrane between the lamina propria and the epithelium, and therefore cells enter the epithelium from an external source. Cell transfer studies failed to show any marked migration of cells from any of the sources studied, and it is suggested that this could be due to manipulation of the cells in vitro. However, a few cells did migrate to the epithelium following injection of spleen, bone marrow, and particularly mesenteric lymph node cells, and therefore the population may be of complex origin.

Once in the epithelium, the cells must either migrate back into the lamina propria, or be shed into the lumen. There are three lines of evidence for the former. Firstly, the recirculating long-lived lymphocytes/

lymphocytes in the thoracic duct lymph are derived mainly from the intestine, and the long-lived population in the epithelium could be one of the sources of these cells. Secondly, cell counts along the length of the villus show that there is a decrease in the number of lymphocytes in the epithelium in the distal third of the villus relative to the central portion. Since the majority of the cells are situated close to the basement membrane, and since no cells were observed crossing the brush-border in this region, then it must be assumed that this decrease is due to the migration of cells into the lamina propria. Finally, electron microscope studies presented evidence of lymphocytes crossing the basement membrane, the direction of migration being most easily interpreted on morphological grounds as a migration from the epithelium into the lamina propria. The presence of lymphocytes in the lacteals suggests that cells may migrate from the lamina propria to other parts of the body.

There is also evidence of cell migration into the lumen of the intestine as well as back into the lamina propria. The proximity of cells to the basement membrane, and the fact that no cells were seen in the process of crossing the brush-border, make it unlikely that there is active migration across the epithelium along the sides of the villus. However, although there is a decrease in the number of cells near the tip of the villus, considerable numbers remain in the epithelium, and these are probably shed passively along with the epithelial cells. It must be remembered that although considerable numbers of epithelial cells are shed into the lumen of the intestine, evidence of active extrusion is not readily observed in sections of proximal intestine, and therefore this could/

could explain the lack of histological evidence of extrusion of the much smaller population of lymphocytes. Direct studies on extrusion, using intestinal perfusion, suffers from the difficulty of classification of the cell types (Pink, Croft and Creamer, 1970). However, Ambrus and Ambrus (1959) found labelled mononuclear cells in intestinal perfusates from dogs injected with labelled leucocytes, and Mitchison (1953) reported the presence of some, but not large numbers of lymphocytes, in perfusates of rabbit intestine. Kotani, Yamashita, Rai, Seiki and Horii (1967) perfused segments of rat intestine, and from their results estimated that over 200 million lymphocytes pass into the lumen each day. Ninety-six per cent of these cells were small or medium sized lymphocytes, the remainder large, an observation which agrees with the nuclear size distribution of the cells in the epithelium found in this study. Thus, there is evidence that lymphocytes are shed into the lumen of the intestine, and the data available suggests that this loss is quite considerable.

The evidence presented in the literature, and that adduced from this study, allows the following conclusions to be drawn about the characteristics of the lymphocyte population in the intestinal epithelium. This population is heterogeneous in its morphology, and in the lifespan of some of its components. The origin of the cells is uncertain. The population is in part self-replicating, but it is probable that cells from several external sources contribute to it. The fate of these cells is also diverse, a small proportion degenerate, some migrate back into the lamina propria, and probably further into the body, and the remainder are lost into the lumen of the intestine. To ascribe any function to these/

these cells, one must necessarily take into account these characteristics. The possible functions are discussed below.

A trephocytic function for lymphocytes, including those in the intestinal epithelium, has been consistently referred to in the literature. Shields, Touchon and Dickson (1969) have been the most recent proponents of this hypothesis, mainly in view of their light microscopic studies which suggested that the lymphocytes in the intestinal epithelium are intracellular, and that large numbers are degenerating. The present study does not support these findings, and therefore it is unlikely that a trephocytic function, in the context of an intracellular degeneration of lymphocytes, is tenable.

Two more modern derivatives of the old trephocyte theory are the reutilization hypothesis, and the hypothesis that lymphocytes are involved in morphostasis. There is now abundant evidence that components of lymphocytes are reutilized to form both new lymphocytes and non-lymphoid tissues (Hamilton, 1956; Fichtelius and Diderholm, 1961; Bryant, 1962; Rieke, 1962; Hill and Spurna, 1968), and it is highly likely that components released from dying lymphocytes are reutilized and contribute in part to the formation of new epithelial cells in the crypts. However, this does not explain the presence of numerous, apparently healthy lymphocytes in the epithelium covering the villi, and it is unlikely that components derived directly from the lymphocytes in situ are being utilized by the epithelial cells.

The second, relatively new hypothesis is that lymphocytes function as tissue growth regulators. Zingar and Svet-Moldavsky (1968) suggested that regulation of certain types of protein synthesis is under lymphocyte/

lymphocyte control. Burwell (1963), and Burch and Burwell (1967) proposed that the primary function of lymphoid tissue is the establishment and maintenance of a steady state condition (morphostasis) in many differentiated tissues, either by lymphocytes, where the target tissue can be freely infiltrated, or by a humoral factor where the tissue lies behind a blood-tissue barrier. Many of the properties of the lymphoid population in the epithelium are compatible with such a hypothesis. Thus, there is the relatively constant ratio of lymphocytes to epithelial cells along the length of the intestine, and in germ-free mice, where the rate of epithelial cell turnover is relatively slow (Abrams, Bauer and Sprinz, 1963; Khoury, Floch and Hersh, 1969), then there are fewer lymphocytes than in conventional mice. Similarly, the change in the metabolism of the intestinal epithelium of weanling mice, in terms of functional enzymes and phagocytosis, is associated with an influx of lymphocytes into the epithelium (see Chapter 5). The presence of two populations of lymphocytes is also explicable in terms of this hypothesis. Such a hypothesis demands a feedback mechanism. The return of some lymphocytes to the lamina propria could be the feedback link to a central lymphoid organ which controls the output of another population of cells, which infiltrate the epithelium, and whose property is to regulate the function of one or more epithelial cells until they are cast off together at the tip of the villus. Such effector cells would thus only need to have a relatively short life span, a property found in part of the lymphoid cell population in the epithelium.

The correctness of this hypothesis must eventually rest on evidence that lymphocytes contain or secrete substances which can influence the/

the metabolism of other cells. Studies with various in vitro models have shown that lymphocytes, or culture fluid supernatants from suitably activated lymphocytes, can inhibit DNA synthesis (Si-Chung, Lein and Klein, 1967; Moorhead, Paraskova-Tchernozenska, Pirrie and Hayes, 1969); exert cytotoxic effects (Ax, Malchow, Zeiss and Fischer, 1968; Ginsburg, 1968; Ruddle and Waksman, 1968; Weiss, 1968; Lundgren and Moller, 1969; Perlmann and Holm, 1969; Williams and Granger, 1969; McLennan and Harding, 1970); cause the inhibition of macrophage migration (Bartfeld and Kelly, 1968; Bennett and Bloom, 1968; David, 1968); stimulate phagocytosis by macrophages (Barnet, Pekarek and Johanovsky, 1968); exert a chemotactic effect (Ward, Remold and David, 1969) and induce vascular permeability (Willoughby, 1969). The term 'lymphokines' has been used to describe this apparent multitude of active substances produced by lymphocytes (Dumonde, Wolstencroft, Panayi, Matthew, Morley and Howson, 1969). Although most of this evidence has been derived from the study of delayed-type hypersensitivity reactions, similar factors may operate in tissue growth regulation in the normal animal as postulated by Burch and Burwell (1967). It may be in fact that cell-mediated reactions to extraneous substances and organisms are an extension of the normal body growth regulatory system.

To summarize the evidence for a trephocytic function for lymphocytes in the intestinal epithelium, changes in the epithelial cell-lymphocyte ratio can be correlated with the changes in epithelial cell turnover rate in conventionalized mice, and with changes in epithelial cell metabolism in weanling mice. This evidence supports the hypothesis that lymphocytes infiltrating the intestinal epithelium are acting as tissue/

tissue regulators. The presence of two populations of lymphocytes, one of which could act as a monitoring or feedback population, and the other as the actual regulating population, is also consistent with this hypothesis. Support for a regulatory function has come from various in vitro studies demonstrating the capacity of lymphocytes to produce substances capable of influencing the metabolism of other cell types.

In contrast to the previous hypothesis, where lymphocytes were postulated to nourish or regulate the epithelium, the presence of large numbers of lymphocytes in the intestinal epithelium has led to the hypothesis that lymphocytes are removing substances from the epithelium during the process of digestion (von Davydoff, 1887; Oppel, 1899; Emerson, 1906; Shields, 1968). The results in this study, and those of other workers have shown that alterations to the diet produce changes in the numbers of lymphocytes in the intestinal mucosa (de Winiwarter, 1930; Meader, 1948). However, since all body lymphoid tissues can be affected by dietary manipulation, and since the lymphoid depletion caused by fasting can be abolished by adrenalectomy (Dougherty, 1959), then stress is probably the major factor in these lymphoid changes rather than any functional response to dietary changes. On this evidence it is concluded that lymphocytes in the intestinal epithelium are not directly involved in the process of digestion.

Since it has been well established that lymphoid tissue is intimately involved in various immunological processes, then it is highly likely that the lymphoid tissue in the intestine is similarly involved. There are three possible roles for the lymphocytes in the intestinal epithelium, that of a central or primary lymphoid organ, that of a humoral or/

or antibody response, or finally, a role in cell-mediated or delayed-type reactions.

The concept of a primary lymphoid organ has been pursued mainly by Fichtelius and his colleagues (Fichtelius, 1967; 1968; Fichtelius, Finstad and Good, 1968; 1969). These workers have proposed that the lymphocytes in the intestinal epithelium are the mammalian equivalent of the avian bursa of Fabricius. The evidence is based largely on phylogenetic studies, and on studies of the kinetics of these cells. The study of intestinal lymphoid tissue in various vertebrates has led them to the suggestion that all vertebrates expressing adaptive immunity have epithelial-associated lymphocytes. The simplest form of this association is a diffuse scattering of lymphocytes throughout the epithelium. In some species, part of this epithelium-lymphocyte relationship has been concentrated into specialized microorgans, for example, in fishes and reptiles, and into the bursa of Fabricius in the avian species. Fichtelius further postulates that even in animals with specialized lympho-epithelial microorgans functioning as bursal equivalents, this activity is supplemented by that of the diffuse epithelial-lymphocyte relationship which is still acting as a central lymphoid organ. Recent studies by Back (1970(b)) would appear to support this concept, since bursectomized chickens have more lymphocytes in the epithelium than controls, findings which can be interpreted as compensatory hyperplasia. Since cell kinetic studies indicate that a proportion of the population is newly-formed, then it is suggested that these newly-formed cells are influenced by the epithelium to perform some function in a way which is comparable to the epithelium of the thymus or bursa of Fabricius. However, the ontogeny of/

of this population is not characteristic of a primary lymphoid organ, and unlike the bursa and the thymus, the size of the population is influenced by the presence or absence of an intestinal flora. The hypothesis of a compensatory hyperplasia following bursectomy is doubtful since bursectomy does have a profound effect on immune responses, and one which would be largely nullified if there was a compensatory hyperplasia of a similarly functioning tissue. On these grounds, and on the finding that the lymphocyte population is decreased by thymectomy (Fichtelius, Yunis and Good, 1968), suggesting partial thymus dependency, then it seems unlikely that the hypothesis of a bursal equivalent is correct.

Although the response of the epithelial lymphocyte population to the presence of a gut flora tends to rule out the hypothesis of a central lymphoid organ, it does suggest the possibility that this population functions in a local defence mechanism. The remainder of this chapter is devoted to discussing the possibility of the involvement of the lymphocyte population in local immune responses, either in terms of antibody production, or in cell-mediated responses.

There is no doubt that immune mechanisms operate across epithelial surfaces. Thus, antigens lead to local antibody production when administered by intramammary injection (Plommet, 1968; Lee and Lascelles, 1970), by intranasal inoculation (Ogra and Karzou, 1969), by aerosol or intratracheal instillation (Leslie and Waldman, 1969), or orally (Thind, 1961; Crabbe and Heremans, 1966; Crabbe, Nash, Bazin, Eyssen and Heremans, 1969; Freter, 1969; Sanyal, Narayanaswami and Mukerjee, 1969; McLeery, Kraft and Rothberg, 1970; Thornard and Aladjem, 1970). Cellular mechanisms can also operate in epithelial surfaces, /

surfaces, as demonstrated by delayed hypersensitivity reactions in the skin (Turk, 1967), but unfortunately, they have not been adequately demonstrated across other epithelial surfaces, with the exception of the colon (Bicks, 1965). However, the observations that lymphocytes from parasitized animals migrate specifically to the gastrointestinal tract of parasitized recipients (Dineen, Ronai and Wagland, 1968), and that lymphoid cells from bronchial washings exhibit specific delayed-type activity in vitro (Henney and Waldman, 1970), suggest that such mechanisms may in fact operate.

It is convenient to look at these local responses in terms of afferent, central and efferent limbs (Miller, 1968). The afferent limb involves the contact of antigen with immunocompetent cells. The central limb involves the subsequent interaction and proliferation of these activated cells, and the efferent limb is the consequence of this proliferative phase, either as antibody production or cell production, which then react with the antigen.

With reference to humoral responses it is necessary, first of all, to outline the general concepts of systemic antibody production before going on to consider the local antibody response. The afferent limb of the humoral response, that is the mechanism by which antigen evokes the central response, is not completely understood. Several different mechanisms may be involved depending on the type of antigen, and whether a humoral or cellular response, or both is being evoked. To stimulate a primary humoral response, there is evidence, for certain antigens, that they have to be processed by phagocytic cells, probably macrophages (Fischmann, 1961; Fischmann and Adler, 1963; Askonas and Rhodes, /

Rhodes, 1965; Gottlieb, 1968; Mitchison, 1969). However, for other antigens, such processing appears to be unnecessary (Unanue, 1969; Shortman, Diener, Russel and Armstrong, 1970). Several component cell types, for example bone marrow and thymic lymphocytes, have been implicated in the central response. This involves the interaction of these different cell types, and includes a proliferative phase leading to the eventual production of the antibody-forming cells which make up the efferent limb. The complexity of this interaction has been the subject of several papers and reviews (e.g. Miller, 1968; Miller and Mitchell, 1968; Mosier and Coppelson, 1968; Abdou and Richter, 1969(a); 1969(b); Coombs and Franks, 1969; Davies, Carter, Leuchars, Wallis and Koller, 1969; Miller and Mitchell, 1969; Nossal, 1969; Richter, 1969; Shearer and Cudkowicz, 1969; Strober, 1969; Brady, 1970).

Applying the above concepts of antibody production to the study of local humoral responses in the intestine, then although the process of digestion involves the breakdown of potentially antigenic molecules, it is now evident that intact antigens can cross the intestinal mucosa. Much of this evidence is indirect in that antibody production resulting from oral administration of antigens implies that they must cross the intestinal epithelium intact (Dubois, Schloss and Anderson, 1925; Hartley, 1942; Farr, Dickinson and Smith, 1960; Miller-Ben Shaul, 1965; Rothberg and Farr, 1965; Uscavage, Gilman and Martin, 1966; Cooper and Thornard, 1967; Rothberg, Kraft and Farr, 1967; Strannengard, 1967; Bernstein and Ovary, 1968; Felsenfeld, Greer and Jiricka, 1968; Mojovic, Andjelkovic and Djordjevic, 1968; Lietze, 1969; Nash, Crabbe, Bazin, Eyssen and Heremans, 1969; Strannengard and Yurchision, 1969; Lefevre, Wilhem/

Wilhem and Raettig, 1970). More direct studies on the fate of antigens, either by analysis of thoracic duct lymph, or in the case of larger molecules, by the study of histological sections, have demonstrated that they can cross the epithelium intact (Alexander, Shirley and Allen, 1936; Bockman and Winborn, 1966; Volkheimer, Schulz, Lehman, Aurich, Hubner, Hubner, Hallmayer, Munch, Opperman and Strauch, 1968; Volkheimer, Schulz, Lindeman and Beitz, 1969).

Since several antigens require processing by macrophages, which are rare in the epithelium, it follows that the lymphocytes there are unlikely to be involved in the afferent limb of a primary antibody response. As a result, the central proliferative phase is unlikely to take place in the epithelium. Antigens capable of stimulating antibody production probably enter the lacteals, and thus progress to the mesenteric lymph node, where it is suggested that they interact with antigen-trapping mechanisms (Mitchell and Abbot, 1965; McDevitt, 1968; Nossal, Abbot and Mitchell, 1968; Diamontstein, Wagner and Grafkeyserlingk, 1969). This consequently leads to a proliferative phase with the formation of antibody producing cell precursors. Since the mesenteric lymph node contains relatively few plasma cells when compared to other active lymph nodes (Olson and Wostman, 1966; Hwang, Sugimura, Ohtaishi and Kudo, 1968; Kotesuikova, 1968), antibody forming cells, or their precursors, must leave the node. The finding that large lymphocytes in the thoracic duct lymph (part of which is derived from the mesenteric lymph node) migrate to the intestine in large numbers, where they are found as pyroninophilic cells, suggests that the efferent limb of the antibody response takes place in the intestine itself. However, /

However, the majority of antibody production occurs in the lamina propria and antibody containing cells are in the minority in the epithelium. In conclusion, the absence of macrophages and significant numbers of plasma cells suggests that the lymphocytes in the intestinal epithelium are unlikely to be involved to any great extent in a local humoral defence mechanism. The hypothesis is presented that local humoral defence mechanism of the intestine has a central component residing in the mesenteric lymph node and an efferent component, that of antibody production, particularly IgA, which functions principally in the lamina propria.

In contrast to the relatively well studied concept of local antibody production, the concept of local cell-mediated responses has been largely ignored. The following discussion is an attempt to advocate such a concept for the lymphocyte population in the intestinal epithelium. Although studies on the fate of contact sensitizers have shown that some antigen enters the draining lymph node (Parker and Turk, 1970(c)), it has been widely suggested that the afferent limb of cell-mediated reactions involves the interaction of immunocompetent lymphocytes with antigen outside lymph nodes. This is the concept of peripheral sensitization (Strober and Gowans, 1965; Brent and Medawar, 1967). The immunocompetent cells involved in peripheral sensitization are thought to be recirculating lymphocytes. Attempts to prove the presence of such a recirculating population in the epithelium by cell transfer studies were largely unsuccessful, but other more circumstantial evidence, which has been presented in this study, suggests that one is present. On the basis of this evidence, it is suggested therefore, that there is a population of/

of immunocompetent cells in the epithelium which can react with antigen in the periphery and then migrate back into the lamina propria.

The next phase in a cell-mediated response is the proliferation of pyroninophilic cells. Studies with skin grafts (Scothorne and McGregor, 1955; Binet and Mathe, 1962), and with contact sensitizers (Oort and Turk, 1965; Wyllie, 1966; Verrier-Jones and Olson, 1969; Wiest and Jung, 1970) have demonstrated that this takes place in the drainage lymph node, which for the intestine is the mesenteric. The presence of dividing pyroninophilic cells in the paracortical areas of the mesenteric lymph node of normal animals may indicate that such a response is taking place, as a result of stimulated lymphocytes entering the node from the periphery and it is suggested that the mesenteric node is the site of the central component of the cell-mediated reaction.

The result of the cell division in the lymph node, in response to contact sensitizers, is the production of lymphocytes which then leave the node (Oort and Turk, 1965; Diengdoh and Turk, 1966; Verrier-Jones and Olson, 1969). These newly-formed lymphocytes presumably migrate to the site of initial antigen interaction. The intestinal epithelium as an interaction site is suggested by the autoradiographic studies in Chapter 4. These show that there is an influx of newly-formed cells into the intestinal epithelium. The cell transfer studies indicate that although the mesenteric lymph node supplies some of these cells, cells from other tissues, including the bone marrow, also migrate to the epithelium. This finding is in keeping with the observation that cells from the bone marrow are involved in the efferent limb of cell-mediated reactions (Liden, 1967; Lubaroff and Waksman, 1968(a); 1968(b); Liden and/

and Linna, 1969). Finally, the labelling index profile of the intestinal lymphocyte population, obtained after a single injection of tritiated thymidine, is similar to that described for delayed hypersensitivity reactions (Kosunen, Waksman, Flax and Tihen, 1963; Kosunen, Waksman and Samuelsson, 1963; Groth, 1967). On the basis of the evidence that there are both recirculating and newly synthesized populations, and that there is a pyroninophilic reaction in the normal mesenteric lymph node, the hypothesis is put forward that lymphocytes in the intestinal epithelium are involved in cell-mediated reactions. It is further suggested that they may be the cellular equivalent of the local IgA system.

Attempts to substantiate this hypothesis by using contact hypersensitivity as an experimental model of cell-mediated reactions were unsuccessful (Chapter 8). However, it is interesting that although delayed hypersensitivity was not induced, tolerance was, and therefore it is suggested that cell-mediated responsiveness was being affected by oral administration of sensitizers, but that under the prevailing experimental conditions, the phenomenon of tolerance was being induced rather than sensitivity. Using a less artificial model, that of conventionalization of germ-free mice, an increase in the number of lymphocytes in the epithelium in response to the presence of a gut flora was shown. On these grounds it is concluded that cell-mediated responses in the intestine have evolved in response to a level of stimulation provided by natural agents, such as bacteria and nematodes, and that experimental studies to date have been on a plane of activity different to that provided by these natural stimuli, and which have resulted in tolerance production.

The/

The whole concept of local immune systems is presented in Figure 9.1. When considering the lymphoid tissue of the villi, then antigens in a form capable of stimulating antibody production probably evoke such a response in the mesenteric lymph node, rather than in the villus itself. The interaction and cell proliferation which follows leads to the production of precursor cells. These enter the thoracic duct lymph and recirculate to the intestine where they differentiate into antibody (IgA) producing cells in the lamina propria. As a result of a considerable migration of cells between different lymphoid tissues (Yoffey, 1967; Ford and Gowans, 1969), other tissues may become involved in what is essentially a local mechanism (Smith, Cunningham, Lafferty and Morris, 1970).

Antigens capable of inducing cell-mediated responses probably do so by interacting with immunocompetent cells in the periphery, that is in the intestinal epithelium. These activated cells then undergo or induce a proliferative response in the mesenteric lymph node, and possibly elsewhere in the body. This ultimately leads to the infiltration of newly-formed lymphoid cells into the epithelium as the efferent or effector limb of the response.

To complete the hypothesis of a local immune mechanism, some account must also be taken of the possible role of the Peyer's patches. Although it has been suggested that Peyer's patches function as a primary or central lymphoid organ (Perey and Good, 1968), in vitro studies have shown that they behave immunologically as peripheral, rather than primary lymphoid tissues (Henry, Falk, Kuhn, Yoffey and Fudenberg, 1970). Since large particles (Joel, Sordat, Hess and Cottier, 1970), and presumably a variety/

variety of other antigens, gain access to the Peyer's patches, then these organs must be subject to continual antigenic stimulation from the intestinal lumen. In this respect, Cooper and his colleagues have shown that the injection of antigen into the rudimentary Peyer's patches of germ-free animals results in histological changes which make the Peyer's patch morphologically identical to those found in conventional rats (Cooper, Thornard, Crosby and Dalbow, 1968). Using similar criteria, Pollard and Sharon (1970) found that the Peyer's patches of germ-free mice only become activated if the antigen is absorbed from the intestinal lumen, and not if it is administered parenterally.

Having established that Peyer's patches respond to antigenic stimulation, it is important to assess their contribution to a local immune mechanism. Analysis of the number of plaque forming cells in Peyer's patches injected with sheep red cells have shown that although there are greater numbers in injected than in uninjected patches, there are considerably less than in the drainage lymph node (Cooper and Turner, 1967). Therefore, antibody production may not be a major function of Peyer's patches. The effect of extirpation of stimulated Peyer's patches on antibody production in other lymphoid tissues, indicates that Peyer's patches seed memory cells to these tissues within 24 hours of contact with antigen (Cooper and Turner, 1969). Thus, the prime function of Peyer's patches may be concerned with the monitoring of the level of antigenic stimulation in the intestine, and the seeding of activated cells to other lymphoid tissues. This hypothesis is further supported by the fact that there is a large scale recirculation of long-lived lymphocytes through the Peyer's patches (Gowans and Knight, 1965), and/

and that lymph draining from them contains about 10 times as many cells as lymph from areas of intestine devoid of Peyer's patches (Baker, 1932). The importance of this diffusion of activity from the Peyer's patches to other lymphoid tissues has been shown by estimating the antibody content of isolated loops of intestine. Thus, loops lacking antigen-injected Peyer's patches secrete as much coproantibody as those containing them, and therefore, direct excretion from the Peyer's patches is not an important factor (Cooper and Turner, 1968).

On the above evidence, the role of the Peyer's patches in the concept of local immunity is probably on the afferent side of the arc. That is, they are probably primarily involved in the induction of the proliferative phase which leads to the production of effector or antibody forming cells. These cells then infiltrate the whole length of the intestine. Whether the Peyer's patches also act in the afferent arc of cell-mediated responses is unknown.

The complete hypothesis, presented in Figure 9.1, is that the afferent limb of local humoral responses is mediated to a large extent focally by the Peyer's patches and mesenteric lymph node. In contrast, the afferent limb of cell-mediated responses occurs in the periphery and is a more diffuse reaction involving the Peyer's patches and the lymphocyte population along the whole of the intestinal epithelium. The central interaction and proliferative phase takes place in the mesenteric lymph node, and to a lesser extent in other lymphoid tissues, and this leads to the production of effector cells. These infiltrate the whole length of the small intestine, the lamina propria in the case of humoral responses, and the epithelium in the case of cell-mediated responses, where/

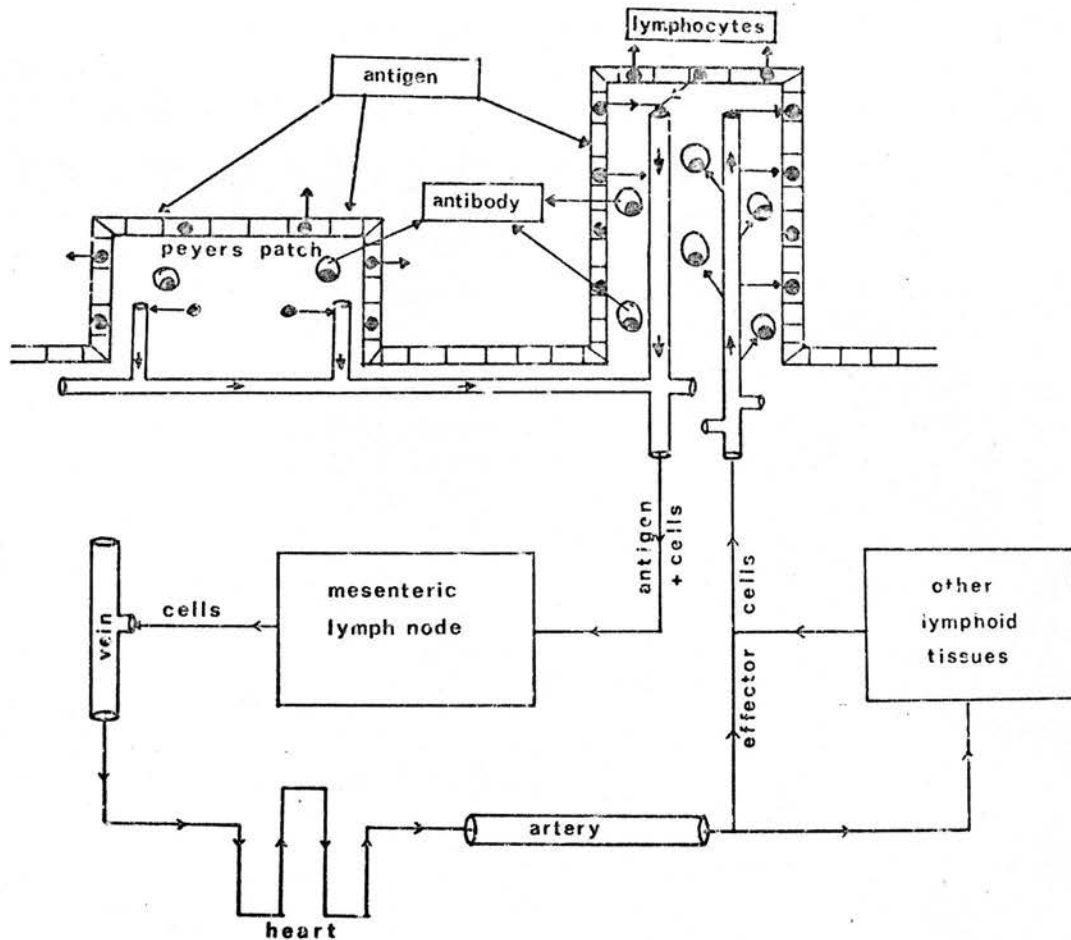


Figure 9.1 Schematic presentation of the cellular pathways in local immune responses.

where they interact with antigen in either the intestinal wall or in the lumen. Since lymphocytes are found infiltrating other epithelia which have similar IgA-producing cells, then comparable local immune mechanisms may operate at most of the body epithelial surfaces in contact with the external environment.

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APPENDIX.MATERIALS AND METHODS.ANIMALS.

The following strains of animals were used.

Mice.

- | | |
|---------|---|
| C3H | - Stock, and also from Animal Suppliers Ltd., London. |
| C57B1 | - Obtained from University of Edinburgh Animal Breeding Centre, Roslin, Midlothian. |
| Ash CS1 | - From Scientific Products Farm, Ash, Nr. Canterbury. |
| CFW | - Bred at Fisons Ltd., Loughborough. |

Guinea-pigs.

- | | |
|---------------|---|
| Porton strain | - Bred at Unilever Research Laboratories or obtained from the Microbiological Research Establishment, Porton. |
|---------------|---|

Rabbits.

- | | |
|-------------|----------------------------|
| Californian | - A.E. Moss, Tring, Herts. |
|-------------|----------------------------|

Housing of Mice.Conventional.

Mice reared under conventional conditions were weaned at 3 - 4 weeks/

weeks of age and placed in groups of 5 or 6 in small plastic cages. Peat moss together with a small quantity of shredded paper was used as bedding. The bedding was changed twice a week. The mice were fed on a commercial diet (Oxoid Rat and Mouse Breeding Diet) and given tap water ad lib.

Germ-free.

Germ-free mice were reared in plastic isolators of a design slightly modified from that of Trexler (1959). Peracetic acid was used to sterilize equipment used in the isolator, and to sterilize the ports following transport of materials into and out of the isolator. Drinking water was sterilized by autoclaving, and food and bedding (wood shavings) by gamma irradiation.

LIGHT MICROSCOPY.

Fixation.

Ten per cent neutral buffered formalin was the fixative used in the earlier work, although Carnoy's fluid was used for the most of the studies. Intestine was fixed by filling the lumen of a freshly removed segment with fixative delivered from a Pasteur pipette, followed by immersion in fresh fixative. This technique prevented the development of air bubbles in the lumen, which often occurred when segments were fixed solely by immersion, and thus ensured more rapid fixation of the villi. This was especially important in fixation of the proximal small intestine.

Processing and Sectioning.

The fixed cylindrical pieces of intestine were dehydrated through alcohols, cleared in cedarwood oil and xylene and embedded in paraffin wax. The blocks were trimmed parallel to the longitudinal axis of the cylinder and 4 μ m - 5 μ m sections were taken approximately half way through the cylinder, at which point the maximum number of longitudinally sectioned villi was obtained.

Staining.

(a) Celestine Blue-Haemalum. This technique was used only on formalin fixed tissues. Sections for cell counting were stained with Celestine Blue-Haemalum, and with Dunn's method for basement membrane as a counterstain. The following staining solutions were used:-

Celestine Blue	(Drury and Wallington, 1967).
Mayer's Haemalum	(Drury and Wallington, 1967).
Dunn's Solution I	- 1% orange G in 1% phosphomolybdic acid.
Dunn's Solution II	- Aniline blue - 0.3 gm.
	Acetic Acid - 4.0 ml.
	Distilled Water - 150 ml.

The staining sequence used is outlined below.

1. Stain Celestine Blue - 5 minutes.
2. Rinse in water.
3. Stain in Mayer's Haemalum - 5 minutes.
4. Differentiate in 1% alcohol.
5. Rinse in water and blue in Scott's Tap Water Substitute.
6. Wash well in running water.

7./

7. Stain with Dunn's Solution I - 15 minutes.
8. Rinse quickly in water.
9. Stain with Dunn's Solution II - 15 minutes.
10. Rinse quickly in water.
11. Dehydrate, clear in xylol and mount in D.P.X.

This technique gives good nuclear staining, outlines the basement membrane and stains the basal portion of the goblet cell cytoplasm orange, thus aiding differentiation between these cells and lymphocytes.

(b) Methyl Green-Pyronin. Several methyl green-pyronin techniques were investigated, and although many of these gave excellent staining of liver and lymph nodes, the equivalent clarity of staining of the intestinal villi was rarely achieved and no method used was consistently good for sections taken from different blocks. The critical point in the staining procedure was the rinse in water and then acetone following the methyl green-pyronin. Excess rinsing in water removed the methyl green, while excess acetone rinsing removed the pyronin. Consequently, the methyl green-pyronin staining was rather a subjective technique, and following the initial staining, the end result was arbitrarily controlled by varying the rinse times in acetone and water until the colour of the sections against a white background appeared pale purple to pink. The following staining solution was the most satisfactory:-

5% Pyronin Y (G.T. Gurr)	4.8 ml.
2% Methyl green (G.T. Gurr)	7.2 ml.
Acetone Buffer pH 4.8	200 ml.
Distilled Water	200 ml

The staining sequence varied according to the fixative used, and followed/

followed approximately the times given below.

	Carnoy	Formalin
1. Methyl green-pyronin	10 - 30 min.	2 - 4 min.
2. Rinse in water	5 - 10 sec.	15 sec.
3. Rinse in acetone (twice)	5 - 10 sec.	15 sec.
4. Acetone/xylol (50:50)	10 sec.	15 sec.
5. Xylol, and mount in D.P.X.		

CELL COUNTING.

Since a great deal of the present work was to involve quantitating the lymphocyte population in the villus epithelium it was essential to derive a reasonably speedy and efficient technique of doing so. To obtain the ratio of lymphocytes to epithelial cells, three methods of quantitation were examined. Since counting the number of lymphocytes in the epithelium was straight forward, the investigation was limited to the more difficult counting of epithelium cells. Only optimally sectioned villi were examined, that is those sectioned perpendicular to the length of the villus, and in such a way that the epithelial cell nuclei appeared as a single row extending along the length of the villus. The count was limited to the area distal to the neck of the crypts. Sections of standard thickness (5 μ m) were used throughout. The three methods examined were:-

(A) Through focus counting. This involved counting all the nuclear fragments observed by focussing from the top of the section through to the bottom.

(B)/

(B) Single focal plane. The number of nuclear fragments in focus and those slightly out of focus were counted using one focal plane only. Using Methods A and B, 5 villi were counted, three times by both methods, to determine the reproducibility of each method and the difference between them.

(C) The villus unit. Using low power objectives, 5 villi were judged to be more or less equal in size and then under oil immersion the number of epithelial cells in the villus unit, that is the area distal to the neck of the crypts, was determined using Method B. Five villi from the proximal intestine of each of five animals of the same age, sex and strain were counted to determine whether or not the epithelial cell count corresponded with the subjectively determined equality of the villus unit.

Results.

Methods A and B.

The results shown in Appendix Table 1 indicated that both Method A (through focus) and Method B (single focus) gave relatively consistent estimates of the number of epithelial cells per villus. However, Method A was extremely slow, and therefore of the two, Method B was to be preferred on the understanding that it underestimated the number of epithelial cells by approximately 20 per cent (19.4 ± 2.3 S.D.).

Method C.

Counts of the epithelial cells in villus units, subjectively determined to be equal, indicated that the range of difference between individual villi could be quite considerable (Appendix Table 2).

However,/

APPENDIX TABLE 1.

Comparison of Method A (through focus) and Method B
(single focus) for counting epithelial cells.

Count number	A (through focus)	Mean	B (single focus)	Mean	Per cent underestimation
1	219		180		
2	216	224	183	183	18.3
3	236		186		
1	248		199		
2	261	257	207	199	22.6
3	262		193		
1	288		224		
2	276	283	228	224	20.8
3	286		221		
1	267		217		
2	262	263	214	218	17.1
3	259		224		
1	256		222		
2	264	261	209	213	18.4
3	262		217		

APPENDIX TABLE 2.

Comparison of epithelial cell counts in villus units
judged to be more or less equal by low power examination.

Villus unit	Mouse number				
	1	2	3	4	5
1	235	249	253	244	281
2	223	217	255	267	210
3	266	268	282	237	204
4	280	293	270	247	299
5	225	231	226	209	252
Mean \pm S.D.	246 \pm 26	252 \pm 30	257 \pm 21	241 \pm 21	249 \pm 42

However, even with as few as 5 villus units per animal, the mean epithelial cell counts were remarkably consistent between different animals so that the number of lymphocytes in the villus epithelium of different animals could be accurately compared in terms of 'n' villus units (providing 'n' is at least 5). This method was very much faster than either A or B but since the dimensions of villi vary in different parts of the alimentary tract and in animals of different ages, the villus unit could not be used with any degree of accuracy to compare animals, unless the animals were approximately the same age, and the same regions of the intestine were being evaluated.

The counting method preferred for studies using the light microscope was therefore that of Method C, where animals were of the same age and where the same regions of the small intestine were being compared. When experiments involved animals of different ages and different/

different regions of the alimentary tract Method B was used and the results expressed in terms of lymphocytes per 1000 epithelial cells.

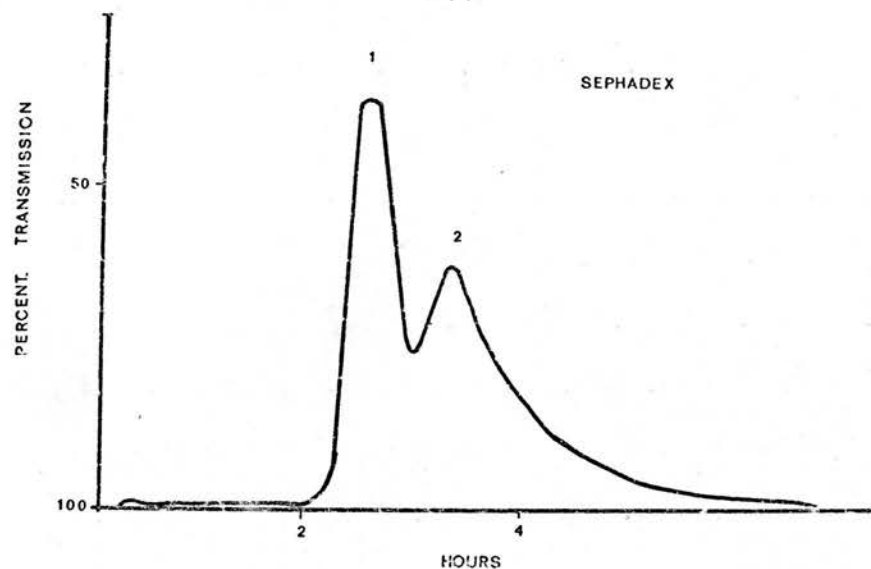
FLUORESCENCE MICROSCOPE STUDIES.

Fractionation of mouse serum.

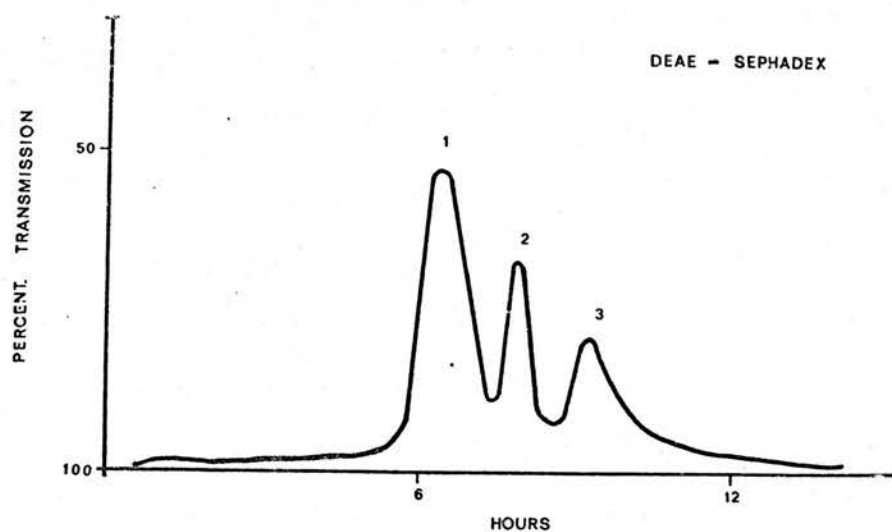
The serum was removed from clotted mouse blood and saturated ammonium sulphate added slowly, with continual stirring, until a final concentration of 40 per cent ammonium sulphate was reached. The mixture was allowed to stand at 0°C for one hour and then the precipitate spun off by centrifugation at 3,000 r.p.m. for 15 minutes. The precipitate was redissolved in 10 ml phosphate buffered saline (PBS), and the ammonium sulphate procedure repeated twice more. The final precipitate was redissolved in 10 ml PBS and then dialysed against two 1.5 litre changes of 0.1 M. Tris. HCl plus 1.0 M. NaCl pH 7.95 for a total time of 20 hours. The dialysed globulin solution was concentrated by ultrafiltration to a final volume of 5 ml.

The globulin sample was placed on a 2.5 x 72 cm G200 Sephadex column (Pharmacia-Uppsala) buffered with 0.1 M. Tris. HCl plus 1.0 M. NaCl pH 7.9 and eluted with the same buffer at a flow rate of 55 ml per hour. Fraction volumes of 12 ml were collected and the protein content estimated by ultraviolet transmission. Two main elution peaks were obtained, the second of which was retained for further fractionation (Appendix Figure 1).

The second G200 peak was concentrated by ultrafiltration and then dialysed against 0.1 M. Tris. HCl pH 8.0 to obtain a sample volume of/



Appendix Figure 1 Transmission curves of Sephadex G200 filtration of mouse globulin solution.



Appendix Figure 2 Transmission curves from further fractionation of Peak 2 from the Sephadex G200 column on DEAE-Sephadex A50.

of 2 ml. The sample was placed on a 1.5 x 30 cm A50 DEAE-Sephadex column and eluted using a linear gradient from 0.1 M. to 0.5 M. Tris. HCl pH 8.0 and a flow rate of 15 ml per hour. Five millilitre fractions were collected and the protein content estimated by ultraviolet transmission. Three main peaks of transmission were obtained (Appendix Figure 2). The fractions corresponding with these peaks were concentrated by ultrafiltration and retained.

The first two peaks were examined by using immunoelectrophoresis and found to contain components of the IgG group of antibodies (Appendix Plates 1 and 2). These fractions were pooled and used to immunize rabbits.

Immunisation of rabbits.

The sample of mouse IgG was emulsified with an equal volume of Freund's complete adjuvant (Difco) by means of rapid passage between two connected syringes. Once the sample was emulsified, rabbits were injected subcutaneously at 5 sites, 3 over the hind quarters and 2 over the fore quarters, with 0.2 ml emulsion. The rabbits were bled 5 - 6 weeks later and the sera examined by immunoelectrophoresis. One rabbit serum produced strong precipitation lines in the gel against mouse IgG (Appendix Plate 3). This serum was retained for use in the fluorescent antibody technique, and the globulin fraction precipitated with 40 per cent ammonium sulphate. The rabbit globulin was not absorbed with mouse light chains and therefore its specificity was directed against mouse immunoglobulin determinants, not specifically IgG.

Appendix
Plate 1

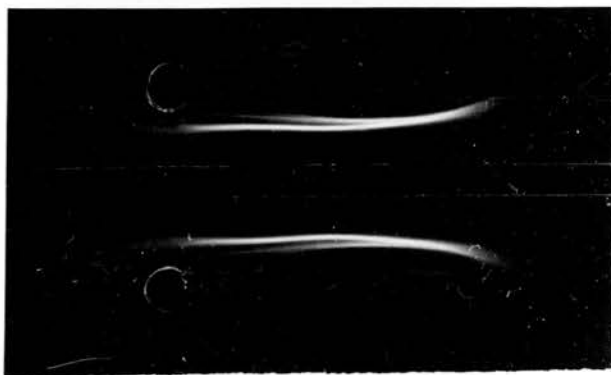
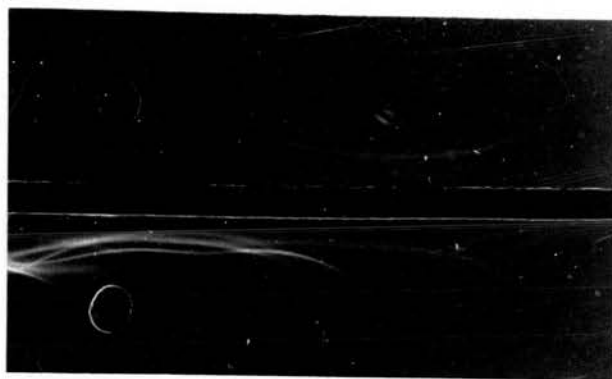
Immuno-electrophoresis of Peak 1 from ion exchange chromatography on DEAE-Sephadex A50. The precipitate corresponds with the IgG precipitate of whole mouse serum.

Appendix
Plate 2

Immuno-electrophoresis of Peak 2 from ion exchange chromatography on DEAE-Sephadex A50. The precipitate corresponds with the IgG precipitate of whole mouse serum.

Appendix
Plate 3

Immuno-electrophoresis of rabbit serum following immunization with Peaks 1 and 2 pooled. The precipitation lines indicate strong anti-IgG activity.



Preparation and staining of sections.

Cylindrical pieces of small intestine were frozen in Arcton 12 (I.C.I.) cooled by liquid nitrogen. Transverse sections, 6 μ m - 8 μ m thick, were cut on a cryostat, mounted on coverslips and fixed for 10 minutes in methanol. After fixation, the sections were rinsed quickly in PBS, and then air-dried with cool air from a hair drier.

The sections were placed in a Petri dish kept moist by means of wet filter paper. One drop of rabbit anti-mouse immunoglobulin was placed on the coverslip and spread over the section using a wire loop. The sections were incubated with the antiserum for 30 minutes at room temperature. After incubation the sections were washed in PBS, gently agitated by means of a magnetic stirrer for 10 minutes, and then air-dried. The dried sections were replaced in the Petri dish and stained for 30 minutes with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Miles Laboratories) diluted 1/8 - 1/10 with PBS. The stained sections were washed in PBS for 10 - 15 minutes and then mounted in glycerol containing 10 per cent PBS for examination under ultra-violet light.

Stained sections were examined on a Zeiss fluorescence microscope with a mercury light source HBO 200W. Exciter filters BG 12/4 plus BG 3/4 plus BG 38/2.5 were used in combination with either barrier filter combination 53/44 or 47/44. Photographs were taken using Ilford HP4 with an exposure time of 3 - 6 minutes.

ELECTRON MICROSCOPY.

Several different fixatives were tried in an attempt to find the most useful one for subsequent studies.

1% osmium tetroxide.

Two buffer solutions were used, 0.1 M. Cacodylate, and Millonig's buffer (Millonig, 1961).

Mice were killed by cervical dislocation. The first 2 cm of the small intestine were removed, and the central portion quickly cut into small pieces 2 x 1 x 0.5 mm prior to placing in fixative for one hour. The specimens were subsequently dehydrated through ethanol, cleared in epoxypropane and embedded in Araldite. One micrometre sections were cut on a Porter-Blum microtome, stained with toluidine blue (Ito and Winchester, 1963) and examined by light microscopy. Thin sections of selected areas were then examined in an AEI 6B electron microscope.

2.5% glutaraldehyde.

Two buffer solutions were tried, 0.1 M Cacodylate, and 0.135 M. phosphate buffer containing varying concentrations of sucrose from 0 - 8 per cent.

A piece of small intestine was removed as above and immersed in fixative. The lumen of the intestine was gently filled with fixative from a Pasteur pipette, and the intestine re-immersed in fresh fixative. The intestine was allowed to fix for $1\frac{1}{2}$ hours. Following fixation, the intestine was cut into small pieces, dehydrated, cleared and embedded in Araldite./

Araldite. In some cases the tissue was washed in buffer and post-osmicated before embedding.

4% formaldehyde.

The following two fixatives were used.

(a) Simple Phosphate Buffer.

NaH_2PO_4 (anhydrous)	3.5 g.
Na_2HPO_4 (anhydrous)	6.5 g.
40% Formaldehyde	100 ml.
Distilled Water	900 ml.

(b) Millonig Buffer.

Solution A	2.26% NaH_2PO_4 .
Solution B	2.52% NaOH .
Solution C	40% Formaldehyde + 5.4% Glucose.
Solution D	41.5 ml A + 8.5 ml B.
Final Fixative	45 ml D + 5 ml C.

The 40% formaldehyde was prepared by dissolving paraformaldehyde in distilled water at 60°C and adding sodium hydroxide until the solution cleared.

A piece of small intestine was removed and immersed in fixative.

The lumen was gently filled with fixative and the tissue allowed to fix for either 10 minutes, $1\frac{1}{2}$ hours or 24 hours. Following formaldehyde fixation the intestine was cut into small pieces and post-fixed in 1 per cent osmium tetroxide, without prior washing, before dehydration through ethanol and embedding.

Choice of fixative.

Using direct osmium fixation, a very common artifact was separation of the epithelium from the lamina propria (Appendix Plate 4). This was so frequent that direct osmium fixation was abandoned.

Glutaraldehyde fixation was sufficiently rapid to prevent detachment of the epithelial layer from the lamina propria (Appendix Plate 5). However, a characteristic feature of glutaraldehyde fixation was the large number of holes in the cytoplasm, especially noticeable in the epithelial cells (Appendix Plate 5). This occurred in all the variations of buffers tried. The large number of holes seen correlated with a decrease in the number of mitochondria stained by toluidine blue, suggesting that the holes represented disrupted mitochondria. This was confirmed by electron microscopy. Post-fixation of glutaraldehyde fixed tissue with osmium tetroxide produced a fine black precipitate distributed uniformly throughout the specimen, and visible only by electron microscopy. This precipitate was not removed by purification of the glutaraldehyde over charcoal, by washing for 24 hours before post-fixation, or by reducing the concentration of glutaraldehyde to 0.5 per cent (Scott, 1970). Glutaraldehyde was therefore discontinued as a fixative.

The results with formaldehyde fixation were more encouraging. Ten minute formaldehyde fixation was not sufficient to prevent the epithelium detaching from the lamina propria when the tissue was handled prior to post-osmication, but $1\frac{1}{2}$ hours and 24 hours gave good fixation as judged by light microscopy. The epithelium was attached to the lamina propria and there were no holes in the cell cytoplasm (Appendix Plate/

Appendix
Plate 4

Osmium Fixation:- Separation of the epithelium from the lamina propria occurred frequently with this method.
Toluidine blue x 150 (approx.).

Appendix
Plate 5

Glutaraldehyde Fixation:- The most common artefact with this fixative was vacuolation in the cell cytoplasm.
Toluidine blue x 1000 (approx.).

Appendix
Plate 6

Formaldehyde Fixation:- This fixative was found to be the most satisfactory. The epithelium remains in close contact with the lamina propria, and the cell structure is relatively well preserved.
Toluidine blue x 500 (approx.).

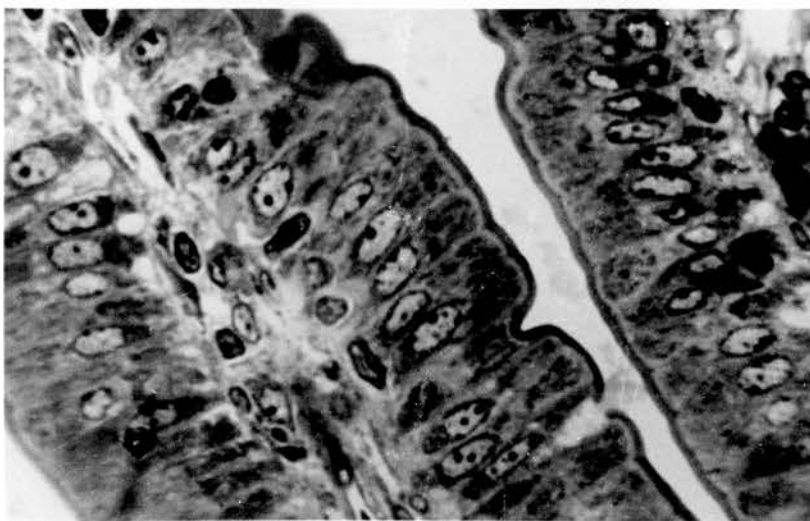
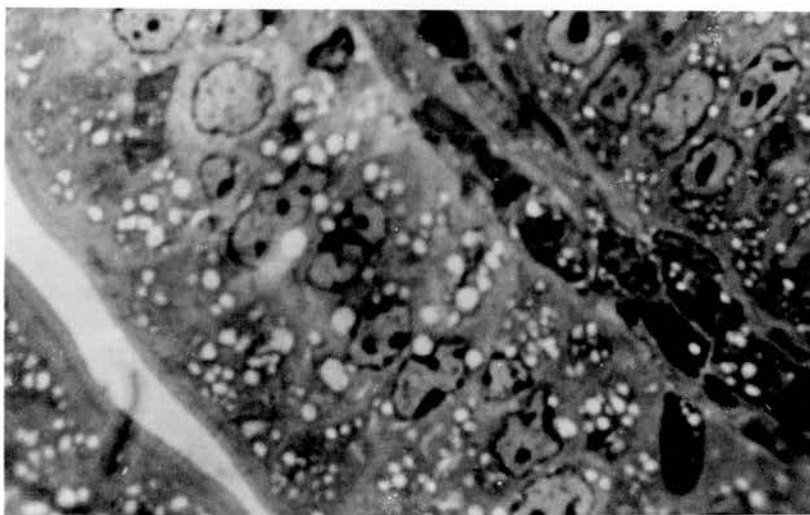


Plate 6). On electron microscopy, both fixatives produced a slight swelling of the cytoplasm, but preservation of nuclear material and the cytoplasmic membranes was reasonable. There was, however, apparent reduction in the granularity of the ground substances of the cytoplasm, and the cytoplasm of 24 hours fixed tissue had a 'washed out' appearance. This was more obvious in tissues fixed in phosphate buffered formaldehyde than in Millonig buffered fixative. Since these disadvantages were of little importance in this study, formaldehyde was the fixative of choice because of its consistent results and reasonable fixation for electron microscope examination.

The following procedure was carried out to prepare tissues for electron microscopy:-

Conventional adult mice 25 - 45 g body weight, outbred and of both sexes were killed by cervical dislocation. A piece of intestine, 1 cm long and 1 cm distal to the pylorus was removed and placed in 4 per cent formaldehyde in Millonig's buffer (Millonig, 1961). The lumen was gently filled with fixative from a Pasteur pipette, and the sample then immersed in fresh fixative for 1 hour. After formalin fixation, the sample was sliced into rectangular pieces 2 x 1 x 0.5 mm, post-fixed in 1 per cent osmium tetroxide for $1\frac{1}{2}$ hours, dehydrated through ethanol, cleared in epoxy-propane and embedded in Araldite. One micrometre sections were cut on a Porter-Blum microtome equipped with a glass knife, and stained with toluidine blue (Ito and Winchester, 1963) for light microscopy. The lower two-thirds of longitudinally sectioned villi was selected for electron microscopic examinations. Thin sections were mounted/

mounted on Athene 483 grids without a supporting membrane, stained with lead citrate and uranyl acetate, and examined in an AEI EM6B microscope.

AUTORADIOGRAPHY.

Mice were killed by cervical dislocation. The piece of intestine between the pylorus and first Peyer's patch (approximately 3 cm) was removed and placed in freshly prepared 10 per cent neutral buffered formalin or Carnoy's fluid. The lumen of the intestine was gently filled with fixative from a Pasteur pipette and the intestine then immersed in a further batch of fixative. When formalin fixation was used, the intestine was allowed to fix for 2 - 3 days and then washed overnight in cold, gently running tap water. After washing, the segment was dehydrated through a series of clean alcohols, cleared in clean chloroform, and embedded in paraffin wax. Tissues placed in Carnoy were transferred to 70 per cent alcohol after 1 hours fixation and then processed without washing. Sections were cut at 5 μ m and floated onto chromic acid cleaned, gelatinized slides. The sections were dried, dewaxed in clean xylol and dehydrated through a series of clean alcohols. The slides were then coated with emulsion. The use of clean glass-ware and freshly prepared chemicals throughout was found to be essential in keeping background on the processed autoradiograms to a minimum.

In the preliminary stages 2 types of photographic emulsion were used, Gevaert NUC. 715 and Ilford K2. The technique of coating the slides with emulsion was identical in both cases. Kodak Wratten No. 1 red safelighting was used with NUC. 715 and Ilford S902 orange-brown safelighting/

safelighting with K2. To prepare the emulsion, 0.5 ml glycerine was mixed with 19.5 ml distilled water to form a diluent which was heated to 43°C in a water bath. A quantity of gel, sufficient to give 20 ml liquid emulsion was placed in a dipping jar (a 100 ml measuring cylinder cut at the 50 ml mark), and allowed to liquify in the water bath at 43°C in total darkness. The heated diluent was then added and the mixture gently stirred. The diluted emulsion was allowed to mix for 5 minutes in the water bath before dipping commenced. To coat the slides, they were removed from the water, drained vertically onto filter paper, and then placed in the dipping jar. The slides were moved up and down 3 times, at approximately 2 - 3 second intervals, withdrawing $\frac{1}{2}$ - $\frac{3}{4}$ of the slide length from the emulsion each time, and then removed slowly and smoothly from the emulsion. The slides were drained vertically for 5 seconds, and the excess emulsion wiped off the back of the slide with tissue paper. The drained slide was then placed horizontally on a cooled inverted aluminium ice-cube tray (one of two alternating in the freezer compartment of a domestic refrigerator), and cooled for at least 2 minutes before being placed vertically against a wooden L-shaped rack to air dry. After air drying in total darkness for 20 - 30 minutes, the slides were placed in sealed light tight black plastic boxes, and exposed, without desiccants, for 2 - 3 weeks at 4°C. A positive control and a fogged non-radioactive slide were included with the experimental slides. Using this technique it was possible to dip 50 slides without heating the emulsion for over an hour.

Following exposure, the slides were developed and stained. Different developers were used for the two types of emulsion, Gevaert 201 developer/

developer for NUC. 715 and Amidol developer for K2. The composition of the developers was as follows:-

Gevaert 201.

Metol	0.75 gm.
Sod. Sulphite (Anhyd.)	25.00 gm.
Hydroquinone	3.00 gm.
Sod. Carbonate (Anhyd.)	16.00 gm.
Potassium Bromide	1.00 gm.
Distilled Water	500 ml.
Developing time	5 - 6 min.

Amidol developer.

Sod. Sulphite (7 H ₂ O)	2.20 gm.
Sod. Hydrogen Sulphite (S.g. 1.34)	0.46 ml.
Amidol	1.00 gm.
Distilled Water	620 ml.
Developing time	7 - 8 min.

Both developers were filtered and used immediately. After developing, the slides were rinsed for 30 seconds in distilled water and fixed in 30 per cent sodium thiosulphate for 10 minutes. The processed slides were washed in gently running tap water for 10 minutes and then stained with methyl green-pyronin.

This preliminary work was designed to find the most suitable emulsion under the prevailing experimental conditions. Ilford K2 was found to be superior because the fewer, large grains per nucleus and the lower/

lower background made microscopical examination of the sections less tiring (Appendix Plates 7 and 8). Other significant factors were the advantages of being able to work in a relatively bright orange light rather than a dark red, and the simpler formulation of the Amidol developer. Ilford K2 was used for all subsequent autoradiography.

Evaluation of slides.

Nuclei with 5 or more grains were considered to be labelled. The epithelium covering the villi, but not that in the crypts, was examined for its content of labelled lymphoid cells. As far as was possible, only longitudinally sectioned villi were counted and lymphoid cells traversing the basement membrane between the epithelium and the lamina propria were counted as being in the epithelium.

Preparation of lymphoid cell suspension.

Mesenteric lymph node and spleen cell suspensions were prepared by gently dissecting the freshly removed tissues with sterile syringe needles, in cold Dulbecco's phosphate buffered saline (DPBS). The resulting cell suspension was passed through a stainless steel filter to produce a single cell suspension.

Bone marrow cell suspensions were prepared from the femur, tibia, ilium and humerus. The bones were cleaned of adherent muscle by rubbing with paper tissue. The epiphyses were crushed with a pair of scissors and the bone marrow gently aspirated with DPBS from a syringe needle inserted in one end of the bone. A single cell suspension was prepared by filtration through a stainless steel mesh.

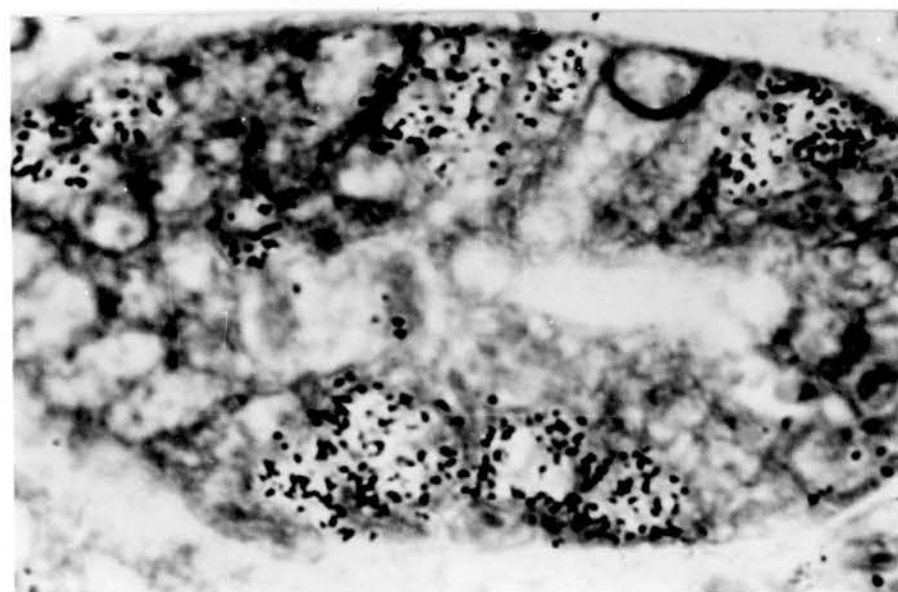
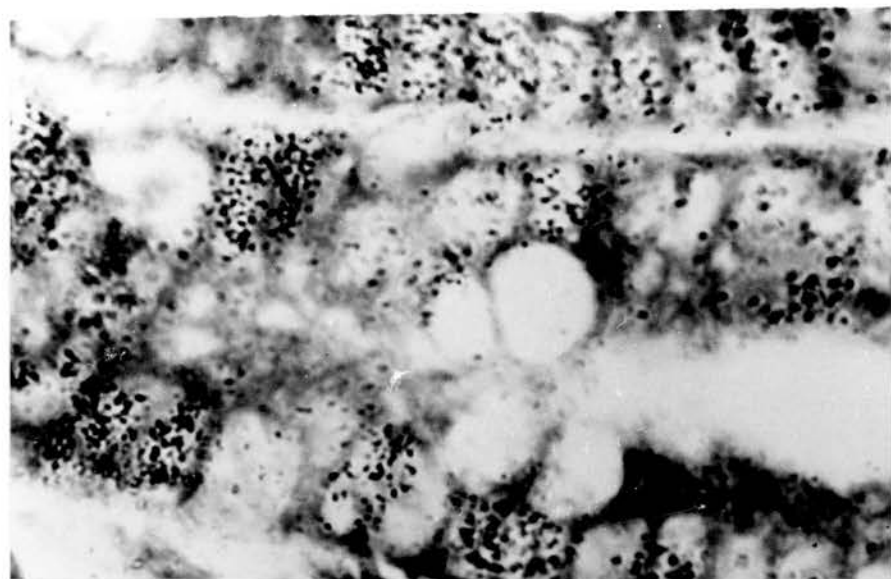
Cell/

Appendix
Plate 7

Labelling of crypt cells following intraperitoneal injection of tritiated thymidine. Thymidine uptake demonstrated with Gevaert NUC 715 emulsion. Methyl Green-Pyronin x 1000 (approx.).

Appendix
Plate 8

Labelling of crypt cells following intraperitoneal injection of tritiated thymidine. Thymidine uptake demonstrated with Ilford K2 nuclear emulsion. Note the presence of fewer larger grains compared with Appendix Plate 7. Methyl Green-Pyronin x 1000 (approx.).



Cell counting was done in a haemocytometer chamber and viability was assessed by Nigrosin exclusion (Kaltenbach, Kaltenbach and Lyons, 1958). The amount of DPBS used in preparing the cell suspension was restricted in order to avoid the necessity of centrifuging a dilute cell suspension to increase the concentration. Cell suspensions prepared in this way usually contained more than 10^7 cells per ml with a viability of over 90 per cent.